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A study of the physical and chemical properties of the insect cuticle

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(*Communicated by Sir William Bragg, F.R.S.—Received* 4 *January* **1940**)

[Plates 1–3]

The morphological, physical and chemical changes in the cuticle during the formation of the puparium in cyclorrhaphous flies are described. The larval cuticle contains about 60% chitin, the puparium about 47%. Almost all the non-chitinous substance is protein. An explanation is given of the hardening of the puparium.

A. Introduction

Among insects, the cuticle (which together with the epidermis constitutes the skin) occurs in two main types: colourless and soft, or pigmented and hard. In general, colourless and soft describe cuticles of larvae of the holometabole insects, hard and coloured those of nymphs of the hemimetabole insects, and of most adults. But the hard and coloured cuticle is always formed colourless and soft, for example, immediately after a moult or in the newly emerged adult. Hardening and darkening take place simultaneously, and start immediately after the moult is completed. Little is known so far about the darkening process, and nothing at all about the nature of the hardening process.

It has been assumed for a long time that the insect cuticle consists essentially of chitin, which, in the case of hard and pigmented types, is associated with pigments and unknown material, called incrustations ('Inkrusten'), which renders it hard. Several treatments have been devised for removing the pigments and incrustations and rendering the cuticle soft and colourless.

Hitherto all attempts to isolate or define the so-called 'incrustation' have failed because of the great resistance of chitin to chemical reagents. Whereas one can remove all the substances associated with chitin, it is not possible to remove the chitin and leave the other substances intact. The obvious way of obtaining information about the physical and chemical processes by which soft and colourless chitin is converted into the hard and dark type, is to compare the cuticle of a freshly moulted insect with the fully hardened and darkened cuticle of the same stage. This method, however, has a drawback in so far as at the time of moulting only the smaller part of the new cuticle already exists, while the larger part is newly formed during the hardening and darkening process (Wigglesworth 1933).

There exists, however, a peculiar case in which a soft and colourless cuticle becomes hard and dark without any apparent growth. This situation occurs in the formation of the puparium in the cyclorrhaphous flies, which include the common houseflies and blowflies. The third larval instar of these flies contracts into the well-known barrel shape of the puparium. When the shaping of the puparium is completed, the cuticle is still white and soft. This stage is commonly called the white pupa, and it is an accurate time mark from which to date the age of the pupa, as it lasts for a few minutes only. Almost as soon as the white pupa is formed, the cuticle starts to darken and harden. The puparium is, therefore, materially identical with the cuticle of the third larval instar, there being no intervening moult as in other cases. It was therefore hoped that a comparison of the physical and chemical properties of the larval cuticle and the puparium would give information concerning the nature of the processes by which this transformation is accomplished and about other material changes which accompany it.

Although the formation of the puparium from the cuticle of the last larval instar is an aberrant case in the insect phylum, the puparium itself is a typical example of the hard and coloured type, while the larval cuticle is a typical example of the soft and colourless type. The results should have, therefore, a general significance beyond the particular reaction which has been the object of investigation.

The present investigation commenced with the blowfly, *Calliphora erythrocephala* Meigen, but later *Sarcophaga falculata* Pand. was almost exclusively used, its much larger size facilitating the preparation of material. The results of this work seem to apply generally in all cyclorrhaphous flies, apart, possibly, from slight differences in the proportion in which the different constituents of the cuticle occur.

The results of the investigation are recorded in the briefest manner in the following pages.

B. PREPARATION OF THE MATERIAL

The larvae were killed by heating them quickly in water at 65° C, at which temperature the muscle protein is coagulated. After cutting them into two parts, the whole content was scraped away from the cuticle with a scalpel. The muscles, which appear white in the coagulated state, are easily distinguished from the colourless cuticle and can be removed quantitatively. Subsequently, except where otherwise stated, the clean cuticles were dried at 100° C and stored in the dry state. Puparia up to the age of 3 days were prepared in the same way. In the early puparium up to 1½ days, removal of the muscles is more troublesome than in the larva, and requires careful cleaning with cotton-wool swabs. In the later pupae, the empty puparia were obtained by pulling out the pupae with a pair of forceps. The use of empty pupal cases, from which the fly had naturally emerged, is not advisable, because they always contain the cast pupal moult, and sometimes part of the meconium. In all the chemical determinations the puparia were previously dried at 100° C. In the X-ray and swelling studies the cuticles were cleaned by scraping, but there was no previous heating of the larvae or pupae. Except in certain special cases the larvae and pupae were reared at 27° C.

C. THE PHYSICAL CHANGES DURING THE FORMATION OF THE PUPARIUM

(1) *Morphological changes during the pupal contraction*

About 1½ hr. before the formation of the white pupa, the larvae become sluggish and very gradually appear to broaden and shorten. This gradual shortening proceeds until the puparium is shaped. This shortening is by no means just a temporary contraction, for during this time the actual extended length of the larva is becoming less and less. The contraction is not reversible. While the larval cuticle has essentially a homogeneous appearance, the puparium shows under slight magnification a regular circular striation which is at right angles to the longitudinal axis. Along these lines of striation the puparium can be readily split.

The shortening of the larval cuticle must have been known for a long time. However, it does not appear to have been realized that the contraction, apart from a muscular action, is mainly a contraction of the cuticle itself. Neither has the phenomenon of contraction taking place in this tissue, which does not contain in itself contractile elements, been regarded as problematical.

Table 1 summarizes the results of several measurements on larval cuticles and puparia. Diagrams showing the relative shapes and sizes of larva and pupa are given in figure 1 (*a*, *b*).

TABLE 1. LENGTH, CIRCUMFERENCE AND AREA OF CUTICLES

	Larval cuticle	Puparium
Length	19–20 mm.	11–12 mm.
Circumference of maximum diameter	12–13	13·5–14·5
Area	160–190 mm.^{2}	102–120 mm.^{2}

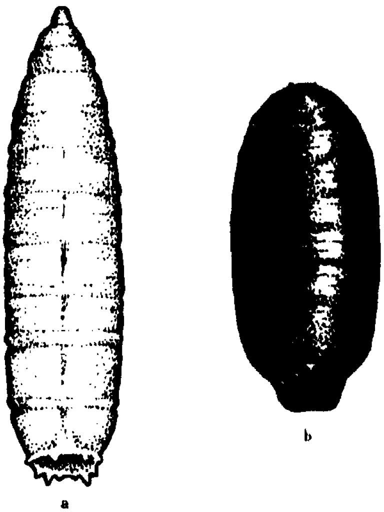

FIGURE 1. (*a*) Larva of Sarcophaga in an extended condition which is obtained by slowly heating the larvae to 60° C. (*b*) Puparium formed by a larva of the same size.

Hence the length of the cuticle contracts *c.* 40 %, while the circumference expands *c.* 12 % during the transformation from larva to pupa. Furthermore, the surface area decreases by *c.* 36 %. It will be shown later that the shrinkage of the larval cuticle on drying amounts to *c.* 22 %. The loss of surface area during pupation is somewhat greater than this, so there is evidence that the structure becomes more compact in the puparium.

(2) *Histological changes during the pupal contraction*

The histological examination of cuticles from *Calliphora erythrocephala* was carried out by Miss B. M. Rickers in the Department of Zoology and Applied Entomology, Imperial College, London. Both ordinary paraffin sections and freezing sections were made. In the latter case, the animals were killed and coagulated by warming at 60° C, but were otherwise not treated with any fixative. Using both these sectioning methods no difference between the thickness of larval and pupal cuticles was detected.

The cuticle consists of two layers, a thin, outer one, the exocuticle, and a thick, inner one, the endocuticle. In the sections cut with the freezing microtome, but not in the paraffin sections, the larval cuticle has a wavy lamellar structure which is confined to the endocuticle (figure 2). Puparia do not have the same structure, but in both the freezing and paraffin sections there is a series of fine lines parallel to the surface (figure 3). When viewed by polarized light, the pupal cuticle appears homogeneous.

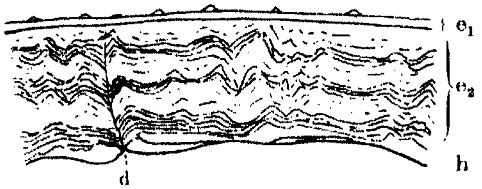

FIGURE 2. Frozen section through the larval cuticle of *Calliphora erythrocephala*, showing the wavy lamellar structure of the endocuticle e^2. e^1 = exocuticle. h = hypodermis. d = duct of a dermal gland.

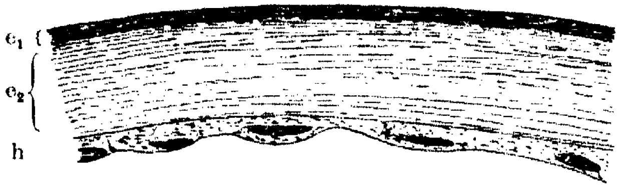

FIGURE 3. Paraffin section through a several hours' old puparium, showing the layers lying parallel to the surface. The exocuticle has darkened.

Immediately after the puparium is formed, darkening of the cuticle sets in and proceeds very quickly. In figure 4 sections of puparia of increasing age are shown. Pigmentation commences at the external surface and proceeds inwards.

(3) *Role played by the nervous system during the formation of the puparium*

It has been shown already (Fraenkel 1935) that ligaturing the blowfly larva across its longitudinal axis does not prevent either half pupating, if this is done less than 16 hr. before pupation. If the ligature is placed a longer period before pupation, only the anterior part pupates, while the posterior part remains unchanged. This is due to the production in the anterior part of a hormone, which is indispensable for the formation of the puparium. The anterior part of the fly larva, separated as it is from the posterior part by a ligature, not only contains the hormone-producing gland, but also the whole of the central nervous system, which is concentrated in the larvae of flies in a single mass in the anterior part of the body. After ligaturing, the anterior part retains its motility, but the posterior part becomes completely motionless.

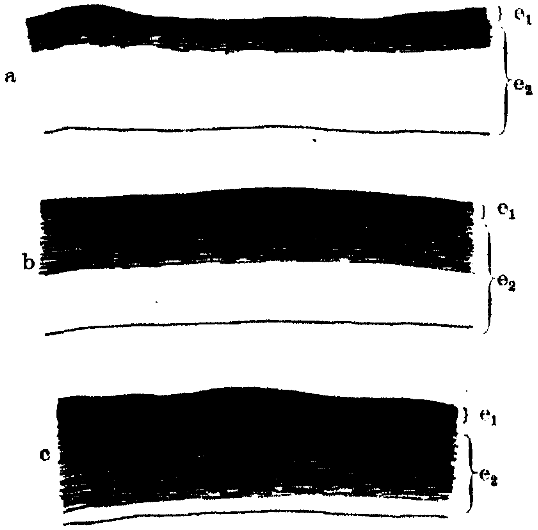

FIGURE 4. The progress of pigmentation in the puparium: (*a*) 1 day old, (*b*) 2 days old, (*c*) 3 days old.

When pupation occurs in the ligatured blowfly larva, the anterior part performs a normal pupal contraction, becomes a white pupa and subsequently darkens. However, the posterior part does not contract, and the cuticle darkens and hardens exactly in the external state in which it is in the larva. This darkening and hardening of the uncontracted cuticle is apparently normal, but the cuticle does not seem to become quite as hard as in the contracted case. From this we obtain two facts:

(1) Darkening and hardening are independent of the contraction process.

(2) The contraction of the cuticle is performed with the aid of muscular processes. This is clear from the fact that if the muscles are paralysed by separating them from the central nervous system, the pupal contraction in the cuticle is no longer possible.

(4) *Changes in molecular structure*

Changes in the molecular structure of the cuticle, during the larva-pupa transformation, were studied by X-ray means. Whereas it was hoped that information concerning both the chitin and non-chitinous substances would be obtained in this way, only the changes occurring in the chitin structure were elucidated. If the non-chitinous substance were protein, it is scarcely to be expected that it would give a pattern that could be distinguished from the much stronger chitin photograph. Certain principal reflexions to be expected from fibrous proteins (namely, 5·1, 3·4, 4·65 and 9–11 A) would correspond very closely indeed with the strongest reflexions in the chitin pattern.

Chitin has been studied by X-rays on several occasions. Meyer and Pankow (1935) have given a reasonable interpretation of the structure, and they find a rhombic cell with $a = 9{\cdot}40$ A, $b = 10{\cdot}46$ A, $c = 19{\cdot}25$ A. The interpretation is based on the close similarity of the chitin and cellulose chains composed as they are of chitobiose and cellobiose units respectively. As a structural framework in the plant cell wall, chitin may replace cellulose, as in fungi (Phycomyces). That chitin has but one crystalline structure, whether of plant or animal origin, has been shown by van Iterson, Meyer and Lotmar (1936) (see also Astbury and Bell 1939).

Clark and Smith (1936) have studied chitin from the lobster (*Homarus americanus*), and also various derivatives of chitin. They make the generalization that chitin in surface structures, such as the carapace, shows no orientation of chains with respect to an axis in the surface, while chitin in fibrous structures, such as the limb 'tendons', is oriented with reference to the long axis of the structure. However, this generalization does not hold in the case of pupa cuticles described in the present paper. With regard to the synthesis and secretion of chitin we know little, but Clark and Smith (1936) draw attention to Borodin's observations on the production of tiny ellipsoidal particles in the cuticle of a marine worm, and refer to these as 'macromolecules' of chitin.

The above-mentioned studies were made on purified chitin, and were aimed, of course, at determining the structure of the chitin molecule. In the present work, the foremost interest has been the study of the chitin-protein complex which constitutes the cuticle. Only a few studies have been made on specially purified chitin obtained from blowfly cuticles.

Preliminary studies were made on material from *Calliphora erythrocephala* but later a more extended examination was made of *Sarcophaga* cuticle. Where similar studies were made, the result was the same in the two types, but the photographs described in this paper were all taken using the *Sarcophaga* material. Cuticles were studied from several stages of the larva, from the white pupa stage and from the later brown pupa stage. They were removed from freshly killed animals, which had not been treated in any way, and the muscles and hypodermis were thoroughly scraped away. In the first place cuticles were studied while still moist. Several layers, cut from the middle region of the segments, were superposed so as to form a bundle of layers, approximately 1 mm. thick and 1 mm. wide. In each case, X-ray photographs were taken, (*a*) with the beam directed perpendicularly to the cuticle surfaces, (*b*) with the beam directed parallel to the cuticle surfaces and parallel to the original longitudinal axis of the body, and (*c*) with the beam directed parallel to the surface and parallel to the transverse axis of the body. For (*c*) strips were cut parallel to the longitudinal axis and were superposed to form bundles similar to those described above.

A description of the diffraction patterns is given below. In these cases are illustrated only photographs which demonstrate certain special points concerning the arrangement of chitin in fresh, moist cuticles. A more complete series of photographs of dried cuticles is given later. Many features of the molecular orientation found in the fresh cuticle remain the same in the dry cuticle.

(1) *Larva.*

(*a*) *Beam perpendicular to the surface.* The diffraction pattern obtained is that of chitin, and consists of a series of Debye-Scherrer rings. From this it can be concluded that there is no orientation of the chitin chains with respect to an axis in the surface (cf. figure 9, plate 1).

(*b*) *Beam parallel to the surface and parallel to the longitudinal axis,* and (*c*) *beam parallel to the surface and parallel to the transverse axis* (figure 5, plate 1). These two orientations of the specimen give similar pseudo-fibre photographs indicating that the chitin chains lie approximately parallel to the surface of the cuticle. That they are not strictly parallel to the surface is shown by the considerable angular dispersion of the 3·4 A meridional arcs.

(2) *White pupa.*

(*a*) *Beam perpendicular to the surface.* The chitin pattern is now oriented with the chains running approximately transverse to the long axis of the body. Apart from the change of orientation, there is no apparent difference between the chitin of the larva and that of the white pupa (cf. figure 11, plate 2).

(*b*) *Beam parallel to the surface and parallel to the longitudinal axis.* From the decreased length of the 3·4 A arc it is seen now that the chitin chains lie more closely parallel to the surface than in the case of the larva cuticle (figure 6, plate 1).

(*c*) *Beam parallel to the surface and parallel to the transverse axis.* The beam is now directed along the average direction of the chitin chains, but there is much dispersion of these about the transverse axis in the plane of the surface. The diffraction pattern shows a kind of superposed ring and fibre photograph, and there is no evidence for the special orientation of side chains, as described later for the dried material (cf. figure 13, plate 2).

(3) *Hard brown pupa.*

(*a*) *Beam perpendicular to the surface.* As in the white pupa, the chitin chains are oriented approximately transversely. This orientation parallel to the transverse axis is a little more marked than in the case of the white pupa cuticle (cf. figure 14, plate 2).

(*b*) *Beam parallel to the surface and parallel to the longitudinal axis.* The chitin chains lie very closely parallel to the surface of the puparium, and this parallelism is more perfect than in the white pupa (cf. figure 15, plate 2).

(*c*) *Beam parallel to the surface and parallel to the transverse axis.* The same general features are observed in this pattern as in the pattern from the fresh white pupa cuticle with the beam directed similarly (cf. figure 16, plate 2).

From the foregoing results of an X-ray examination of cuticles, the following conclusions are to be drawn:

(1) In the larval cuticle, the chitin crystallites lie only approximately parallel to the surface, and they are disoriented in planes parallel to the surface.

(2) In the white pupa the chitin crystallites lie more closely parallel to the surface, but the most striking feature is that they now show orientation roughly parallel to a transverse axis in the surface.

(3) In the hard brown pupa the crystallites are oriented still more closely parallel to the surface than in the white pupa, while the orientation parallel to a transverse axis in the surface is also a little better developed.

These results are in complete agreement with observations on the microscopic structure of the cuticles. In the larval cuticle transverse striations are not seen, while these are very typical of the white pupa cuticle and the puparium. In the larva, the X-ray diagrams with the beam directed parallel to the cuticle surface show only an approximate parallelism of the chitin chains with reference to the surface and this must be due in part to the unevenness of the layers as seen in perpendicular sections of the larva cuticle (figure 2). The cuticles of all stages can be split into layers parallel to the surface, and this is just what would be expected from the arrangement of chitin chains. That the puparium can be readily split along the transverse axis is, of course, a direct consequence of the orientation of the chitin chains approximately parallel to the transverse axis.

In the transformation from larva to white pupa, the pupa cuticle appears to contract at the same time as the body is contracted by muscular action. Whereas in the temporary contractions of the motile larva the cuticle folds, during the formation of the white pupa the cuticular contraction is effected by a change of molecular orientation. Such a change is one which would be expected to occur if there were a process of stretching directed along a transverse axis in the surface. The lateral expansion of the contracted body immediately suggests itself as a cause. Measurements made upon the circumference of larvae and pupae (see p. 4) show an increase of 12 % in the circumference of the pupa. This, together with the tension developed during the removal of water following the white pupa stage, would account for much of the orientation of the crystallites so that they lie approximately parallel to the transverse axis. To illustrate this, a section from the cuticle of a larva was stretched laterally by 12 % and allowed to dry at this new length. Upon taking an X-ray photograph with the beam directed perpendicularly to the surface, it was seen that the chitin chains lie approximately parallel to the direction of stretching, and have a degree of orientation approaching that found in the normal brown puparium. The degree of orientation of the chitin in the puparium explains the effect observed, namely a contraction of 40 % in the length of the cuticle. The primary cause of this effect is considered to be the lateral expansion of the contracted body, but there may be other factors which influence the change of molecular orientation in the intact cuticle.

To illustrate the mechanism of transverse orientation in another way, we may discuss the result of studies upon cuticles of ligatured larvae. The effect of ligaturing has been described in a previous section, and it is only necessary to recall that the normal contraction of the larva is prevented, although the process of pupation proceeds more or less normally. X-ray photographs of these hard brown cuticles were taken as in the case of normal puparia, but the material was allowed to dry in air.

(*a*) *With the beam perpendicular to the surface* (figure 7, plate 1). The diffraction pattern obtained shows no orientation of the chitin chains with respect to an axis in the surface. This result differs markedly from that found in the corresponding hard brown cuticle of normal non-ligatured pupae.

(*b*) *With the beam parallel to the surface and parallel to the longitudinal axis* (figure 8, plate 1). The pattern shows that the chitin chains lie approximately parallel to the surface. In addition, as the reflexions of spacing 4·65 A (200) and 9·5 A (002) are at right angles to one another, it is clear that the (002) planes tend to lie parallel to the surface. In figure 18A, (002) planes contain both the axes *a* and *b*, and it is the planes *ab* which lie parallel to the surface. This particular orientation is due to the flat shape of the crystallites as illustrated in figure 18D.

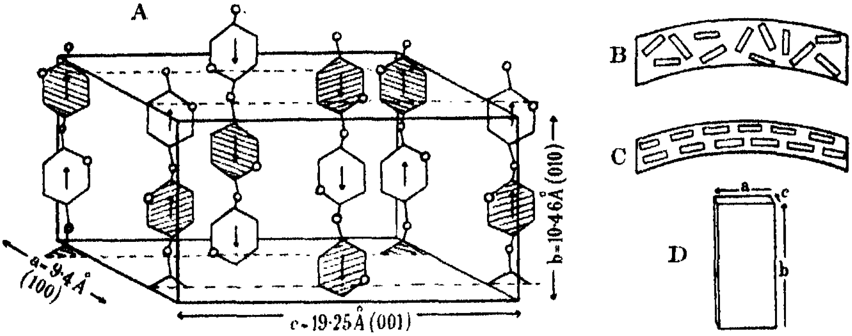

FIGURE 18. A, unit cell of chitin as found by Meyer and Pankow (1935). B and C, vertical sections of puparium cut parallel to the longitudinal axis. B before purification: C after purification. The diagrams show the random dispersion of the *a* and *c* axes in the natural material B (cf. figure 16) and the orientation of the *a* axis parallel to the surface in the purified material C (cf. figure 17). D, diagram of the chitin crystallite showing the laminar form.

From these X-ray studies it is evident that the transverse orientation of chitin chains in normal puparia is dependent upon the normal contraction of the body by muscular action. The peculiar condition in the puparium of ligatured larvae, where (002) planes of the chitin structure lie parallel to the surface, may be explained by contraction forces developed in the plane of the surface during drying. Water is removed from the cuticle while it is stretched over the body, which remains in its extended condition.

Information upon the kind of aggregation of chitin and protein was obtained from a study of the effect of drying cuticles. Photographs taken of cuticles, dried in air upon the surface of glass plates, gave the following results:

(1) *Larva.*

(*a*) *Beam perpendicular to the surface* (figure 9, plate 1). The diffraction pattern is in all general appearances similar to that of fresh, moist cuticles. However, one difference between the photographs of wet and dry specimens is apparent immediately. In the case of wet specimens, the reflexion at 9·5 A (002) is displaced towards the centre, but this effect may be due to the presence of proteins, in which structures the side-chain spacing is similar and is susceptible to the action of water. The effect is observed in all stages from larva cuticle to puparium, and it can be seen in a comparison of figures 6 and 12.

(*b*) *Beam parallel to the surface and parallel to the longitudinal axis* (figure 10, plate 1). The chitin chains lie approximately parallel to the surface, the parallelism being closer than in the case of moist cuticles. In addition, as the reflexions 4·65 A and 9·5 A are at right angles, it is clear that the (002) planes have become oriented parallel to the cuticular surface. An essentially similar diffraction pattern is obtained when the beam is directed parallel to the transverse axis.

(2) *White pupa.*

(*a*) *Beam perpendicular to the surface* (figure 11, plate 2). The chitin orientation is very similar to that in the moist pupa cuticle.

(*b*) *Beam parallel to the surface and parallel to the longitudinal axis* (figure 12, plate 2). The chitin chains lie closely parallel to the surface, as observed in the case of the wet cuticle.

(*c*) *Beam parallel to the surface and parallel to the transverse axis* (figure 13, plate 2). In this pattern it is evident from the position at right angles to one another of the 4·65 A (200) and 9·5 A (002) reflexions that the (002) planes tend to lie parallel to the surface.

(3) *Hard brown pupa.*

The results obtained are practically identical with those using moist puparia. There is no definite evidence that (002) planes become oriented parallel to the surface in the dry material. Photographs taken with various orientations of the specimens are given below:

(*a*) *with the beam perpendicular to the surface* (figure 14, plate 2);

(*b*) *with the beam parallel to the surface and parallel to the longitudinal axis* (figure 15, plate 2);

(*c*) *with the beam parallel to the surface and parallel to the transverse axis* (figure 16, plate 2).

These observations on the orientation of (002) planes during drying of the larva cuticle provide some information on the state of aggregation of the chitin at this stage. If the chitin crystallites were firmly linked together, it is unlikely that they would show this change of orientation upon drying. It seems, therefore, that the micelles or crystallites are relatively separated, or at most very loosely attached to one another. While no similar observation can be made upon dried puparia because of the very small contractions which occur on drying, the state of aggregation of the chitin was studied in the following way. Puparia were compressed between glass plates in water and in steam by applying strong pressure with Hoffman clips. An X-ray examination of such specimens showed very little orientation of the crystallites with the (002) planes lying parallel to the surface. The orientation remained substantially the same as it is in the uncompressed condition. On the other hand, larva cuticles pressed while fresh show almost complete orientation of (002) planes, while the effect of pressing white pupa cuticles shows a similar change of orientation, but this is less complete than in the larva. In the fresh larva cuticle the *a* and *c* axes are randomly dispersed in planes perpendicular to the surface, but in the dried or pressed cuticles the *c* axis lies perpendicular to the surface, while the *a* axis lies parallel to the surface. Heyn (1936) finds this condition in the cell wall of sporangiophores of the fungus *Phycomyces*, but as his studies have been made upon pressed and presumably dried material, it is not certain that the same orientation would be found in the living cell wall. In their studies of soluble chitin precipitated from solution in hydrochloric acid, Clark and Smith (1936) observed no orientation of crystallites with the (002) planes lying parallel to the surface. However, in their chitosan prepared from a chitin sheet, such as the lobster carapace, they observed that the (002) planes of the rhombic chitosan lie approximately parallel to the surface. The treatment with a saturated solution of sodium hydroxide at 95° C, as used to produce the chitosan, seems to have freed the crystallites so that the *a* axes rotate and lie parallel to the surface of the dried sheet.

In our investigations a similar observation was made on puparia which were purified in diaphanol, a treatment which results in substantially pure chitin. After such treatment, the crystallites have rotated so that the (002) planes lie parallel to the surface, as is shown clearly in figure 17, plate 3, where the beam is directed parallel to the surface and parallel to the transverse axis (cf. figure 16, plate 2). One interpretation of this phenomenon would consider the removal of the intermicellar material from around individual micelles, thus allowing freedom of rotation of crystallites. The binding effect of this intermicellar substance is clearly seen in the resistance offered to compression forces, no appreciable orientation of the (002) planes being effected. In figure 18, plate 3, A represents the unit cell of chitin, as found by Meyer and Pankow (1935), while B and C give the orientation of chitin crystallites with reference to the plane of the surface in normal and purified puparia. D illustrates the approximate shape of the laminar chitin crystallite.

It is apparent that the chitin is more dispersed in the larva cuticle than in the puparium. Whereas the crystallites can be rotated in any direction in the larva cuticle, this freedom of rotation is lost in the puparium, where the chitin appears to be aggregated into a more rigid framework. In a later section measurements will be given of the swelling of cuticle from larvae and pupae, and these throw further light on the factors which determine the hardness of the puparium.

Comparison of diffraction patterns obtained from larval and pupal stages

In order to be certain that the crystalline structure is the same in every detail in two specimens, diffraction patterns were compared by the sector method described by Astbury and Woods (1933). By this method one can detect small changes in spacing and small variations in the intensity and width of the lines. To be certain that varying orientations are not the cause of the observed differences, only specimens ground to powder with pestle and mortar were employed. Such powders were packed into holes to give equal thickness of material in the direction of the transmitted X-ray beam.

A complete series of comparisons was made between material from the various stages, both before and after purification. The more important of these are described and figured below:

(*a*) *Comparison of cuticles from larva and white pupa stage.* In figure 19, plate 3 it can be seen that the chitin pattern is practically identical in larva and white pupa cuticle. The spacings as well as the intensities of the various lines correspond exactly.

(*b*) *Comparison of white pupa cuticle and the brown puparium* (figure 20, plate 3). Whereas the spacings of the chitin still correspond in the two cases, the intensities of some of the lines differ. In powder photographs some of the observed reflexions are superposed, and this applies to the [(030) (031)], the [(002) (100)], the [(200) (004) (201)], the [(012) (110) (014)] and to the [(202) (210)] group of reflexions. In the brown puparium those reflexions which are comparatively unaltered are (020), [(030) (031)] and (040). Those that appear distinctly more diffuse are [(002) (100)], [(012) (110) (014)], [(200) (004) (201)], [(202) (210)], (203), (223), (230).

Thus we can conclude that in the puparium the crystallinity of the chitin has become less perfect along the *a* and *c* axes, with no comparable effect along the *b* axis. A similar change was observed when white pupa cuticles were compared with puparia obtained from ligatured larvae.

(*c*) *Comparison of the brown puparium with chitin from purified puparia* (figure 21, plate 3). The purified chitin was obtained by treating puparia with diaphanol. For comparison with the natural puparium, this chitin was mixed with protein extracted in water from larva cuticles. The mixture was made up of one part of chitin to one part of protein, these being very nearly the proportions occurring in the natural puparium. The comparison photograph shows that reflexions other than (0*k*0) are altered in the natural puparium, but not in the mixture.

(*d*) *Comparison of purified white pupa chitin with natural white pupa cuticle* (figure 22, plate 3). The chitin was obtained pure by treatment with 5 % potassium hydroxide solution. It was mixed with approximately 40 % of the larval cuticle protein to give a mixture resembling closely the composition of the natural cuticle of the same stage. In this comparison no marked difference between the chitin patterns was detected other than a slight weakening and diffuseness of the (203) and [(202) (210)] reflexions in the photograph of the natural cuticle.

(*e*) *Comparison of purified white pupa chitin and purified brown pupa chitin.* From figure 23, plate 3, it can be seen that there is no difference between the purified chitin from these stages.

From this series of comparisons we can conclude that the *purified* chitin is essentially the same in all stages. It has been demonstrated, however, that at the hard brown pupa stage the chitin in situ undergoes a change, the crystallinity apparently deteriorating along the *a* and *c* axes, but not along the *b* axis. The alteration is not permanent, since on removal of the impregnating material the degree of crystallinity returns to about that observed in the white pupa and larval stages. Changes in chitin structure as brought about by the action of concentrated hydrochloric acid (Clark and Smith 1936), show first a progressive disappearance of the (030), (031) reflexions and then a reduction in the perfection of the (002) reflexions. The (200) spacings remain unaffected. The alteration observed in the transformation to the brown puparium is in the opposite direction, the (200) and (002) reflexions being changed while the (030) is unaffected.

The most probable mechanism of this change at the brown pupa stage is by way of association between chitin and protein. Astbury, Preston and Norman (1935) have given evidence for a mixed crystallization of cellulose and xylan in vegetable fibres, the two substances having a fundamentally similar structure. The natural fibres yield X-ray photographs which are interpreted as showing less perfect crystallinity than xylan-free fibres. In the blowfly cuticle we have some kind of a chitin-protein combination, and on account of the general spacial similarity between chitin and protein of the α-keratin type, a mixed crystalline condition is conceivable. A large body of information is growing to show the important inter-relationship between polysaccharides and protein, and one can specially instance immunity reactions. Also, in the widely occurring mucoproteins we have various carbohydrates constituting the prosthetic groups (Meyer 1938).

The observed change of chitin structure occurs in the hard brown puparium. Combination between chitin and other substances must alter the physical properties of both materials, and it is most likely that the hardness of the puparium can be attributed, in part, to such changes.

(5) *The swelling of the cuticle*

The hardness of a tissue, such as the cuticle, will be dependent upon the degree of hydration of its structure. In a later section are given estimates of the water content of larva and pupa cuticles, but here we are concerned principally with the dimensional changes of cuticles upon drying.

Small rectangular pieces of cuticles of known orientation were measured with the microscope while wet. They were then allowed to dry upon the surface of mercury at 65 % relative humidity and 22·2° C. Some of the specimens were replaced in distilled water and the swelling was measured. The mean values for the contraction on drying and the swelling on rewetting are given below:

TABLE 2

	Percentage contraction from fresh to dry state		Percentage swelling from dry to rewetted state	
	along transverse axis	along longitudinal axis	along transverse axis	along longitudinal axis
Larva	13·5	10·0	8·1	7·2
White pupa	8·2	11·6	5·3	9·2
Puparium (light brown)	3·1	4·3		
Puparium (hard brown)	0·94	2·5		

From table 2 it is clear that during the larva-pupa transformation the cuticles progressively lose their ability to swell and hold water. From studies upon the fresh and dry weights of cuticles (p. 18) it is seen that there is approximately three times as much water in the larval cuticle as in the 1½ day puparium. This change in the water content is undoubtedly connected with the change from the soft and pliable condition of the larva and white pupa cuticle to the hard and brittle puparium.

The swelling of the chitin itself was estimated from a study of a hard brown puparium which had been purified in diaphanol.

From tables 2 and 3 it is seen that the swelling of the chitin framework and of brown pupa cuticle are approximately of the same order. Hence the protein content of the puparium makes no apparent contribution to the amount of swelling.

TABLE 3

Percentage contraction from wet state to dry state	
Along transverse axis	Along longitudinal axis
2·2	3·6

The picture which can be drawn from these data is as follows: chitin in the larval cuticle is in the form of a dispersed phase in a highly hydrated protein phase. In the brown puparium the degree of hydration has been greatly reduced, and chitin and protein are aggregated into a firm apparently continuous framework. In a previous section, evidence has been given for a combination of protein and chitin, and it seems that the puparium is effectively such a continuous phase of chitin-protein. In the puparium the protein fails to swell. This may be due to the condition of the protein itself, or merely because it is enclosed in a continuous framework of chitin. Evidence for the comparatively loose structure of the chitin in the larva cuticle and the more compact structure in the puparium has been given in the previous section.

D. THE CHEMICAL CHANGES DURING THE FORMATION OF THE PUPARIUM

(1) *Increase in dry weight*

It is clearly shown in table 4 that the dry weight increases considerably in the puparium. Since the weight of the cuticles of larvae reared at different times varies considerably, each series of determinations has to be treated by itself. In each series care was taken that only larvae or pupae of approximately equal size were chosen, and for each determination of a series, between ten and twenty larvae or pupae were used.

The puparium reaches a weight which is 122–130 % that of the cuticle of the white pupa. Most of this weight increase takes place during the first day of the puparium. The weight also increases considerably from the moment the larva is fully fed and starts to empty the gut until the formation of the white pupa.

TABLE 4. DRY WEIGHT IN MG. OF CUTICLES DURING THE COURSE OF DEVELOPMENT (EXPRESSED AS WEIGHT OF SINGLE SPECIMENS)

	Series I	Series II	Series III	Series IV	Series V
Larva, crop full	3·3	—	7·2	—	—
Larva, crop empty	5·3	6·0	—	8·8	9·5
White pupa	5·1	6·1	9·1	9·2	10·2
Pupa, 1 hr.	5·3	—	—	—	—
Pupa, 4 hr.	5·7	—	—	9·8	—
Pupa, 2–12 hr.	—	—	11·8	—	—
Pupa, 8 hr.	5·7	7·2	—	—	—
Pupa, 1 day	6·6	—	—	—	—
Pupa, 2 days	6·0	—	—	—	—
Pupa, 3 days	6·1	—	11·3	—	—
Pupa, 10 days	6·4	7·9	11·1	11·7	13·2
Pupa, 10 days as % of white pupa	125 %	130 %	122 %	126 %	130 %

(2) *Change in water content*

For determining the water content, fresh cuticles were blotted thoroughly on filter paper, weighed, dried at 100° C, and weighed again.

TABLE 5. DRY WEIGHT OF THE CUTICLE OF VARIOUS STAGES, EXPRESSED AS PERCENTAGE OF WET WEIGHT

	Series I %	Series II %	Series III %	Series IV %
Larva	27·8	32	—	29
White pupa	—	33	—	32
Pupa, ½ hr.	—	—	—	43
Pupa, 1 hr.	—	41	—	—
Pupa, 4 hr.	—	50	—	—
Pupa, 5 hr.	—	—	45–48	—
Pupa, 8 hr.	—	—	—	57
Pupa, 1½ days	—	63	—	60
Empty puparia, air dry	88·8	—	—	—

Table 5 shows that puparia during the first hours undergo a rapid water loss and this action only starts after the white pupa is formed. The determinations were not systematically continued in pupae older than 1½ days. At about this period the hypodermis becomes separated from the puparium, which undergoes further drying until, presumably, it is in equilibrium with the atmospheric humidity. In its early stages, the puparium is in contact with the living tissues and undergoes considerable changes through activity of the hypodermal cells with which it still forms a unit. If the pupae are kept at a saturated humidity, water is lost in precisely the same way as with normal pupae. This is shown in a series where white pupae were placed in 100 and 0 % humidity and kept there for 5 hr. at 27° C. At the end of this period the dry weight of those kept at 100 % humidity was 45·2 %, and of those kept at 0 % humidity 48·4 %. The difference between these figures seems insignificant.

TABLE 6. SOLUBILITY OF CUTICLES OF VARIOUS STAGES IN WATER AND IN 5 % KOH AT 100° C (PERCENTAGE RESIDUE GIVEN)

	Series I Treated with		Series II Treated with		Series III Treated with		Series IV Treated with	
	Water at 100° C for 2 days %	KOH 5 % at 100° C for 2 days %	Water at 100° C for 1 day %	KOH 5 % at 100° C for 2 days %	Water at 100° C for 1 day %	KOH 5 % at 100° C for 2 days %	Water at 100° C for 1 day %	KOH 5 % at 100° C for 2 days %
Larvae crop full	68·8	57	—	—	—	—	68·1	53·9
Larvae crop empty	—	—	—	—	63·7	59·3	67·3	55·2
White pupa	69·7	59·5	68·8	58·5	64·7	56·5	64·4	54·4
Pupa 1 hr.	—	—	—	—	—	—	69·9	54·0
Pupa 2 hr.	—	—	75	56	—	—	—	—
Pupa 4 hr.	—	—	—	—	—	—	73·4	51·5
Pupa 3–12 hr.	78·9	51·2	—	—	—	—	—	—
Pupa 6 hr.	—	—	78·1	51·2	75·6	49·8	—	—
Pupa 8 hr.	—	—	—	—	—	—	76·7	48·9
Pupa 16 hr.	—	—	—	—	—	—	81·4	48·1
Pupa 1 day	—	—	—	—	—	—	89·3	46·8
Pupa 1½ days	—	—	87·7	45·6	—	—	—	—
Pupa 2 days	—	—	—	—	—	—	93·8	48·6
Pupa 3 days	*96·5	48·7	—	—	—	—	94·9	49·8
Pupa 4 days	—	—	96·4	48	—	—	—	—
Pupa 7 days	—	—	95·3	48·5	92·2	47·7	92·9	47·2
Pupa 14 days	89	46·8	92	46	—	—	—	—

* One day's treatment.

(3) *Changes in solubility of certain constituents of the cuticle during the formation of the puparium*

The primary aim of these studies was to determine accurately the amount of chitin present in the cuticles of different stages. Samples of cuticles of different stages were treated first with water at 100° C. for 24 or 48 hours, and then with 5 % potassium hydroxide solution at 100° C. for 48 hours. Repeated experiments and checks with different methods showed that 1 or 2 days' treatment at 100° C. decreased the weight of the cuticles by an amount which is subsequently only slowly altered by further treatment. The results of several series of determinations are tabulated (Table 6). Each series refers to a separate batch of larvae of identical size, and the cuticles were prepared at various subsequent stages of development. It is seen that the amount of water soluble material decreases steadily from about 33 % in the larval cuticle to about 8 % in the puparium. At the same time, the amount of alkali soluble material, which includes the water soluble fraction, increases from 40% in the larval cuticle to 53% in the puparium. The changes in the amount of water-soluble and alkali-soluble material are comparatively small during the conversion of the larva into the white pupa. The major changes with respect to solubility occur during the first day of the pupa, which is a period in which the puparium is still in contact with living tissue.

(4) *Constancy of chitin*

(*a*) After treating larval cuticles or puparia with 5% potassium hydroxide solution at 100° C, approximate constancy of weight is reached in 1–2 days. Residues as a percentage of the original weight are given below:

Larval cuticle: 62·6, 62·5, 61·9, 61·6, 59·3.
Puparia: 47·6, 47·9, 46·9, 46·8, 46·3, 47·2, 46·0.

(*b*) Cuticles treated with 5% hydrochloric acid gave the following results:

Larval cuticle:	1 day in 5% HCl at 100° C	59·8%
	2 days ,, ,,	57·4%
Puparia:	1 day in 5% HCl at 100° C	55·4%
	2 days ,, ,,	50·3%
	4 days ,, ,,	45·2%

The above figures are slightly lower than those obtained by other methods. The low residue weight in the case of larval cuticles may be correlated with the tendency of these to break into small fragments when treated in this way.

(*c*) As a third solvent a concentrated solution of chlorine dioxide in 50% acetic acid was used. This solution is known as diaphanol. Puparia treated at room temperature (18° C) gave the following residue weights:

1st week	71·7%
2nd week	53·2%
3rd week	49·4%
4th and 5th weeks ...	47·9%
6th and 7th weeks ...	47·4%
8th and 9th weeks ...	47·2%

After 10 weeks' treatment in diaphanol, larval cuticles reached constancy of weight at a figure which is 60·4% of the original weight.

It is suggested that the residue in all these cases is pure, or almost pure, chitin, and support for this view is given by the following facts:

(1) The nitrogen contents of purified cuticles were determined.

Larval cuticles treated with 5 % KOH N-content = 6·4 %
Puparia treated with diaphanol N-content = 6·5 %
6·3 %
Puparia treated with 5 % KOH N-content = 6·5 %
6·4 %

The theoretical figure for the N-content of chitin is 6·89 %.[*]

(2) Chitin is soluble in concentrated mineral acids. The residue after acid, alkali or chlorine dioxide treatment dissolves almost completely in concentrated hydrochloric acid in the cold.

We can, therefore, regard the chitin content of the larval cuticles to be about 60 %, and that of the puparia at the time of emergence about 47 %. Considering that the dry weights of puparia and larval cuticles are related approximately as 125:100, it appears that the absolute amount of chitin is practically unchanged from the larva to the pupa. The proportion 60:125 is very nearly equal to 47:100. The same result has been obtained more directly. The entire cuticles of larvae and puparia in different stages were treated for 2 days with 5 % potassium hydroxide solution at 100° C. The weight of the insoluble matter, which is considered to be chitin, is given in table 7. The figures in brackets indicate the number of specimens used for the determinations. In series II and IV the larvae were obtained from batches in which the specimens were smaller than those which provided the material of series I and III.

TABLE 7. THE AMOUNT OF CHITIN PER LARVA OR PUPARIUM (IN MG.)

	Series I	Series II	Series III	Series IV
Larva crop full	4·07 (20)	1·8 (20)	—	—
Larva crop empty	—	2·9 (20)	6·1 (10)	3·6 (21)
White pupa	5·47 (16)	2·75 (12)	—	3·5 (15)
Pupa 3-12 hr.	5·66 (15)	2·8 (15)	—	3·6 (15)
Pupa 3 days	5·5 (10)	3·0 (20)	—	—
Pupa 14 days	5·23 (20)	3·0 (20)	6·2 (10)	3·75 (20)

It is seen that the amount of chitin in cuticles of larvae (crop empty) is practically identical with that found in puparia. The increase of weight from the larva with the crop full to the white pupa is the expression of a real growth of chitin, and this has been dealt with already, tables 4 and 6.

The conclusion is that the amount of chitin remains unchanged during the formation of the puparium and the subsequent hardening processes. This result is particularly noteworthy in view of the fact that hitherto the hardening of chitin has often been spoken of as 'chitinization' and a hard sample of insect cuticle as being 'heavily chitinized'.

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[*] We are indebted to Mr A. R. Trim for making the nitrogen determinations reported in this paper.

(5) *The protein fraction of the cuticle*

There are many indications that the water- and the alkali-soluble fractions of the cuticles consist almost entirely of protein.

(*a*) From the previous sections it is seen that about the same fraction as is soluble in dilute alkali is also soluble in diluted mineral acids.

(*b*) The nitrogen content of the cuticles has been determined as 9·8 % in larvae and as 10·9 % in puparia. Therefore we can calculate the nitrogen content of the alkali-soluble fractions with the help of the following data:

N-content of insoluble chitin = 6·4 %
Chitin content of larva cuticle = 60 %
Chitin content of puparium = 47 %

Hence the nitrogen content of the alkali-soluble fraction both from larval cuticles and puparia is 14·9 %. This value is typical of proteins.

(*c*) The water-soluble fraction shows strong protein reactions. The amount of water-soluble material is much greater in the larval cuticle than in the puparium. As the treatment is protracted, or the temperature raised, more material dissolves in water, table 8.

TABLE 8. THE AMOUNT OF WATER-SOLUBLE MATERIAL IN LARVAL CUTICLES AND PUPARIA, AS A PERCENTAGE OF THE TOTAL WEIGHT

Kind of treatment	Larval cuticle	Puparia (old)
1 hr. at 100° C	14·8	2·1
1 day at 100° C	24·3, 23·7, 26·1	7·8, 8·1, 6·0
2 days at 100° C	31·4, 32·7	11·0, 10·6
5 hr. at 135° C	33·8	18·2

(*d*) The nitrogen contents of the dissolved substances obtained by autoclaving at 135° C for 5 hr. were determined as:

15·2 % N for larval cuticles.
15·1 % N for puparia.

These figures, which are very similar to those calculated in (*b*) above, suggest that the whole of the extract may consist of protein.

Concerning the nature of the proteins in water extracts, some preliminary tests were carried out, with the results shown in table 9.

It appears from the various tests reported in table 9 that the reactions of the protein, dissolved from the puparia on autoclaving at 135° C are very similar to those of the protein dissolved from the larval cuticles treated at 100° C for 1 day. That there are changes in the protein fraction is clear from its reactions with acetic acid. When extracted in the cold, it does not precipitate with acetic acid; extracted at 100° C it precipitates partly, and when extracted from the puparium by autoclaving, it precipitates entirely. A detailed investigation of the characteristics of proteins in the larval cuticles and puparia is at present in progress.

TABLE 9. PROTEIN TESTS CARRIED OUT WITH WATER-SOLUBLE FRACTION OF THE LARVAL CUTICLES AND PUPARIA

Treatment	Millon reaction	Trichloroacetic acid 5 %	Ammonium chloride, half-saturated	Acetic acid, 1 %
		Larva cuticles		
Water extract in the cold, after grinding with sand	+	Precipitates, redissolves on heating	Precipitates, redissolves on diluting, not on heating	No precipitation
Water extract at 100° C for 1 day	+ +	Precipitates, redissolves on heating	Precipitates, redissolves on diluting, not on heating	Precipitates, redissolves in excess acid, or on heating
		Puparia		
Water extract in the cold, after grinding with sand. Nothing dissolves	—	—	—	—
Water extract at 100° C for 1 day	+	Precipitates, redissolves on heating	Precipitates, redissolves on diluting, not on heating	Precipitates, redissolves in excess acid and on heating
Water extract at 135° C for 5 hr.	+ +	Precipitates, redissolves on heating	Precipitates, redissolves on diluting, not on heating	Precipitates, redissolves on heating and in excess acid

The protein in the larval cuticle, which is largely water-soluble, becomes in the puparium insoluble, the greatest solubility change being shown after the white pupa stage. Linked with this is the great change in water content and ability to swell in aqueous solutions. The change in solubility may be similar to that shown in the relationship between collagen and gelatin, gelatin being produced from collagen on autoclaving. However, the cuticular protein differs from gelatin in giving an intensely positive Millon reaction and in precipitating from aqueous solution with trichloroacetic acid.

X-ray diffraction patterns of proteins extracted from larval cuticles in the cold, and of extracts at 100° C, are typical of a system of fully extended polypeptide grids. It should also be noted here that the patterns indicate that the cuticular protein does not actually belong to the collagen-gelatin group (see Astbury 1938).

(6) *Graphical presentation of the changes in solubility of the various constituents of the cuticle during the larva-pupa transformation* (*figure* 24)

The measurements during the pupation process of change in cuticle weight, loss of water, change in solubility of the proteins in water, and change in the proportion of chitin to non-chitinous matter, can be presented in a graphical form which illustrates the interrelationship of the changes. The absolute amount of chitin is unchanged. The water-soluble fraction (24 hr. treatment at 100° C) decreases rapidly during the first day of the puparium, and slightly more until the third day, after which time it remains constant. The water-insoluble, alkali-soluble fraction increases rapidly during the first day of the puparium, and slightly more until the third day. It is clear that the greater part of the water-soluble protein of the larvae becomes water insoluble in the puparium. However, it seems to be present in the puparium in a state in which it can be made soluble again by treatment at high temperatures. The whole of the fraction, constituting the increase of weight from the larval cuticle to the puparium, is alkali-soluble. It consists of pigments and, according to the evidence of the previous section, protein.

✓ (7) *The pigmentation process*

(*a*) After the white pupa is formed, it immediately starts to darken, and passes through a yellowish and a reddish to a deep brown state.

(*b*) Figures for the tyrosine content of larval cuticles were 2·38 and 2·37 %, and for the puparia 1·92 and 1·93 %.[*] Taking into account the increase of weight shown by the puparium and assuming that this increase carries no tyrosine, then the amount of tyrosine in the puparium, supposedly free from the weight increase, is 2·40 %. Thus in the pupa, all the tyrosine originally present in the larval cuticle can be accounted for.

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[*] We wish to thank Dr Malpress for making these determinations using Lugg's method.

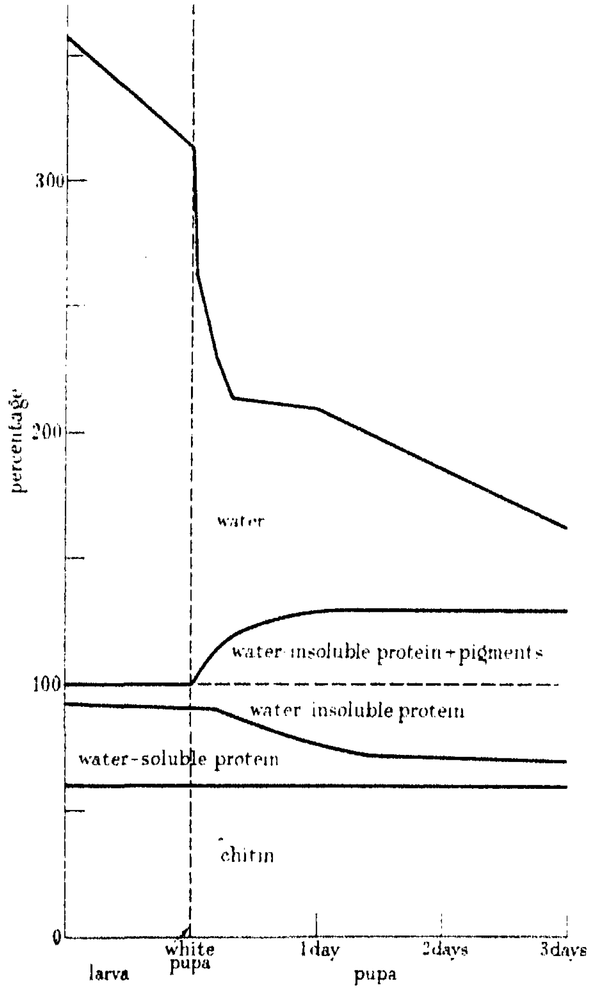

FIGURE 24. Graph showing changes in water content, weight, and solubility of various constituents of the cuticle of Sarcophaga, from the late larval stage until the emergence of the fly. Abscissae = time in days. Ordinates = percentage weight with reference to that of the white pupa cuticle which is taken as 100 %.

(c) The tyrosinase-tyrosine reaction requires the presence of free tyrosine. Actually, no free tyrosine could be found in the water-soluble fraction of the larval cuticles. This supports the view that tyrosine in the pigmentation process is derived from within the body.

(*d*) The water extracts of the larval cuticle do not show the presence of 'dopa' with the ferric chloride test, but the 3–6 hr. old puparia give a marked positive reaction. It is concluded that no free 'dopa' is contained in the larval cuticles.

(*e*) Since the origin of pigment in the puparia is entirely from outside the cuticle, the chromogen being derived from inside, and oxygen from the atmosphere by way of the tracheae, the pigment constitutes an addition to the weight of the larval cuticle. The nitrogen content of melanin itself has been found to be about 8·6 % (Raper and Wormall 1925). The nitrogen content of the non-chitinous substances in the puparium is about 15 %. Assuming that this is protein nitrogen, we may conclude that melanin is present in very small quantities and contributes little to the increase of cuticle weight after the white pupa stage.

E. Discussion

Hitherto it has been generally assumed that soft and colourless types of insect cuticle consist almost entirely of chitin, and that hard and coloured types contain, in addition to pigments, unknown substances called incrustations. The presence of lipoid material in the cuticle was first recognized by Kühnelt (1928), but since this substance constitutes only a very small fraction, we are neglecting it for the present. The presence of protein has been shown qualitatively by Kühnelt (1928) and Wigglesworth (1933), but it is only recently that the large proportion of protein in the cuticles has been recognized (Evans 1938).

The difficulty of determining the constituents of the insect cuticle has arisen from the fact that three, possibly four, largely insoluble substances —namely, chitin, melanin, insoluble proteins, and possibly another undetermined substance—are contained in the cuticle in close association. In selecting the cuticle of the blowfly larva and its conversion into the puparium as the object of the present investigation, it was hoped that the physical and chemical changes which it undergoes during this conversion would throw light on the composition of both states in which the insect cuticle occurs; namely, the colourless and soft type, and the coloured and hard type. It has been pointed out in the Introduction how it is that larval cuticles and the puparia constitute particularly favourable material for the study of these problems. On the other hand, it must not be forgotten that we are dealing with a somewhat aberrant case, and that not all the results can be applied immediately to other objects. It will be partly the object of the following paragraphs to consider the general validity of the results obtained.

(1) *Quantitative determination of the chitin content of tissues*

Hitherto two main methods have been employed for determining the amount of chitin in the insect cuticle. Van Wisselingh's chitosan method, in the modification described by Campbell (1929), consists of heating the cuticle in a saturated solution of potassium hydroxide at 160° C for 30 min., during which time all the substances which are associated with chitin dissolve, and the chitin is converted into chitosan. As a quantitative method for the estimation of chitin this procedure is not likely to yield reliable results, since the chemistry of chitosan is not sufficiently well known.

Considering these difficulties, Campbell uses this method mainly as a qualitative test for chitin, and for quantitative purposes he treats the cuticle in 15–20 % potassium hydroxide solution at 60° C for 14 days, after which time the residue is assumed to be almost pure chitin.

The other method of purifying chitin is by treatment in a concentrated solution of chlorine dioxide in 50 % acetic acid. This reagent, introduced under the name diaphanol by Schulze (1922), was originally employed for purifying cellulose from lignin and other incrustations with which it is associated in wood. Koch (1932) claims that treatment of insect cuticles with chlorine dioxide until complete decoloration has taken place, followed by treatment with sodium bisulphite, removes the incrustations quantitatively. With this method, the chitin content in the elytra of *Melolontha vulgaris* has been found to be 48 %, in the cuticle of the caterpillars of *Pieris brassicae*—64 %, in the abdominal sclerites of *Periplaneta orientalis*—38 %. We have shown that treatment of puparia for 9 weeks with chlorine dioxide yields a residue which is almost pure chitin. However, there can be no doubt that short period treatment with diaphanol, as it is employed in the histological technique, removes only a small part of the non-chitinous substances. The slowness of the purifying action of chlorine dioxide is probably an advantage, in that it leaves the chitin fraction substantially unchanged.

In our experience 2 days' treatment of cuticles with 5 % potassium hydroxide solution at 100° C yields practically pure and unchanged chitin. By this treatment, about 40 % substance is removed from the larval cuticles, and 53 % from the puparia of Sarcophaga. Recently, Evans (1938) reports that with the cuticles of the meal worm, *Tenebrio molitor*, 65·9 % dissolved in 1 % potassium hydroxide during 2 hr. treatment at 100° C. The nitrogen content of the alkali-soluble part was 15·4 %, and of the residue 8·5 %. This treatment was not carried through till constancy of weight of the residue was attained, and the nitrogen value of the residue suggests that the state of pure chitin was not reached. Therefore, the chitin content of the meal worm cuticle must be regarded as well below 34 % of the cuticle. The same treatment with the cuticle of the blowfly larva, *Calliphora erythrocephala*, yielded 49 % residue, with a nitrogen content of 7·1 %. The nitrogen content of the extracted material suggested to Evans that it was chiefly protein, and this conclusion was confirmed by the strong positive xanthoprotein and Millon reactions and the weak biuret reaction. The large quantities of protein in the insect cuticle revealed by the publication of these data had not been expected.

Buxton (1932) described a method for determining the amount of cuticular material in insects. This consisted of drying whole insects, grinding them up and treating them in potassium hydroxide solution for 24 hr. The residue was taken to represent the skeletal tissues, or, in other words, the cuticle. From Evans' observations, and also from our own experience, it is clear that this treatment removes much alkali-soluble material from the cuticles, and the figures obtained represent considerably less than the amount of the entire cuticle, and considerably more than the amount of chitin. It includes, incidentally, all material which does not dissolve in 1 % potassium hydroxide at 100° C within 24 hr., irrespective of its cuticular or non-cuticular origin.

(2) *The pupal contraction*

The formation of the puparium has often been referred to as a pupal contraction. It is indeed initiated and brought about by contraction of the body muscles. After disconnecting the central nervous system by ligaturing the larva at its anterior end, no contraction takes place during the formation of the puparium. The mechanism of the change of dimensions in the cuticle has been elucidated by X-ray diffraction studies. The cuticular contraction proceeds by changes of the orientation of long chitin crystallites from their arrangement in the larva, where they are randomly dispersed in planes parallel to the surface, to their arrangement lying approximately parallel to the transverse axis in the puparium. In the case of ligatured larvae, the arrangement of the chitin chains in the pupa is approximately the same as in the larva. The simplest explanation of this rearrangement is to consider a lateral expansion of the contracted pupa body, in which case the cuticle is stretched laterally, and, in fact, a lateral extension of the isolated cuticle does produce a similar rearrangement of the chitin chains. It is probable that loss of water from the cuticle also causes strains along the transverse axis, and thus improves the orientation. These observations on the contracting cuticle give an excellent picture of the way in which orientation of organic material can be effected. In many cases of relative growth the orientation in one structure must be affected by the growth of a neighbouring tissue.

It is certain that the lateral orientation of the chitin structure plays a part in the mechanism of escape from the puparium. Although the splitting of the puparium during the emergence of the fly takes place along a line of preformed weakness, the transverse orientation of the chitin crystallites must increase very considerably the ease with which this cleavage can be brought about.

(3) *The hardening of the puparium*

Insect cuticle is either soft or hard, and hard cuticles always pass through a soft stage in the course of the hardening process, but it has been suggested that an undetermined substance (incrustation) was the cause of the hardening. Great emphasis was laid upon the analogy between chitin and cellulose, which are closely related polysaccharides. It was assumed that cellulose hardens into wood by the formation and addition of lignin; similarly, incrustations were considered to harden chitin.

During the hardening of the puparium several processes take place which will influence the hardness.

(*a*) The higher degree of chitin orientation in the puparium would increase the tensile strength of the cuticle, and thus contribute to its apparent hardness. The hardness and brittleness of the puparium is, nevertheless, not completely explained in these terms. Compared with puparia purified with diaphanol, the natural puparia are more brittle and give the impression of being harder, but the chitin orientation is similar in both cases. Also, in the puparia of ligatured larvae, the orientation of the chitin remains much as it was in the larva, yet the pupa cuticle of this type is considerably hard.

(*b*) On account of the dehydration process during the first day of the pupa, the substances which compose the puparium become more closely packed together than they are in the larval cuticle. This constitutes another cause of hardening. Although no other investigations have been made on the water content of soft and hard cuticles, it is certain that the process of dehydration during hardening is of general occurrence in insects. It can be assumed that soft cuticles of larvae have a much higher water content than hard cuticles of adults.

(*c*) The water soluble proteins of the larval cuticle become largely insoluble in the puparium. This is possibly due to a condensation of smaller units into larger units. Evans (1938) found that the loss in weight of the cuticle of the adult *Tenebrio molitor* after treatment in potassium hydroxide solution at 100° C is 'very much less than in either pupa or larva'.

(*d*) The ability to swell in water is very largely lost in the hard brown puparium, whether in normal or ligatured specimens. The X-ray studies and the swelling data lead to a picture of the chitin-protein association. In the larva cuticle we have a dispersed phase of chitin in protein, while in the hard puparium we have a condensed chitin-protein association. The chitin lattice is affected, and a mixed crystallization of chitin and protein is suggested. Such a mixed crystallization is likely to prevent protein swelling and to increase the hardness of the structure.

Experiments were described showing that water is removed from the puparium in contact with the body, even in a saturated atmosphere. This suggests that condensation of the chitin structure is squeezing water out of the protein, or as is more probable, that there are changes in the protein itself with the elimination of water and loss of ability to attract water. It is suggested that the dehydration of the cuticle, the growing insolubility of its constituents and the loss of swelling power are due to the protein hydrophilic groups reacting with one another and/or with the chitin side-chains.

The factors enumerated in (*a*) to (*d*) would be sufficient to account for the hardening process and may be summarized as changes in the chitin structure and changes in the protein structure. This kind of phenomenon is probably related to the process taking place during the encystment of unicellular organisms and, as far as the protein constituent is concerned, to the phenomena of keratinization in vertebrates. For example, the teeth of the lamprey, though continually immersed in fresh water, attain a considerable degree of hardness.

It does not seem necessary to postulate the existence of special incrustations which cause the hardness of the puparium, and there is only a restricted meaning in the term 'chitinization' which has been used to describe the process of toughening, or hardening, in insect cuticles. Whereas there is relatively more chitin in the soft larval cuticle, the structure is soft because of the hydration of the protein content. In the puparium there is relatively less chitin, but the protein has lost its ability to swell. Hence the swelling of the puparium is very similar to that of chitin itself and only in this sense can the puparium be referred to as 'chitinized'.

(4) *The pigmentation process*

Dewitz (1902) has shown that the colouring of the puparium of flies is a fermentative process, and since then it has been recognized that the ferment involved is the oxidase tyrosinase, which converts tyrosine into melanin in the presence of molecular oxygen. The presence of 'dopa' as an intermediary stage in the formation of the pigment in the several hours old puparium, and the absence of 'dopa' in the larval cuticle suggest that the pigmentation starts from tyrosine. No tyrosine is contained in the free state in the larval cuticle, and it was shown by an analysis of the total tyrosine contained in larval cuticle and puparium, that the chromogen for the pigmentation process is derived from within the larva. It has been shown previously (Fraenkel 1935) that the oxygen which is required for this fermentative process is supplied through the tracheae, and does not come directly from the atmosphere. In view of the fact that both the chromogen and oxygen are obtained from inside the larva, it is surprising that the colouring starts in the outer layer of the puparium and proceeds inwards.

The authors are indebted to Dr W. T. Astbury for his advice and his interest in the work, and to Dr Malpress and Mr A. R. Trim for making certain chemical determinations. The financing of the research was assisted by the Rockefeller Foundation, and by a grant from the Agricultural Research Council.

SUMMARY

1. The morphological and histological changes in the cuticle during the formation of the puparium in cyclorrhaphous flies are described.

2. The pupal contraction, which results in the barrel-like shape of the puparium, is performed by muscular action. If the central nervous system is separated from the muscles, a puparium is formed retaining the shape of the larval cuticle.

3. During the conversion of the larval cuticle into the puparium the following physical and chemical changes take place:

(*a*) The arrangement of the chitin crystallites changes from random orientation in planes parallel to the surface in the larval cuticle, to an arrangement in the white pupa cuticle and puparium where they lie approximately parallel to the transverse axis. This change of orientation explains the degree of contraction in length shown by the cuticle.

(*b*) In the larval cuticle the chitin crystallites can be rotated in any

direction by the application of forces of compression or extension. This freedom of rotation is lost in the puparium. After compression or simply by allowing the larval and white pupa cuticles to dry on glass plates, the chitin crystallites become oriented so that (00*l*) planes lie parallel to the surface. The same orientation is obtained in puparia only after removal of the non-chitinous substances. It is suggested that these impregnating substances hold the chitin crystallites firmly together.

(*c*) The ability of the larval and white pupa cuticles to swell in water is very much greater than that of puparia, whether normal or from ligatured specimens. The swelling of puparia is of approximately the same order as that of the chitin framework itself.

(*d*) The dry weight of the puparium increases to about 125 % of that of the larval cuticle.

(*e*) The water content decreases from about 70 % in the larval cuticle to about 40 % in the 36 hr. old puparium. This dehydration is regarded as having a chemical basis. Later a further purely physical drying takes place, and in the air-dried puparium there is a water content of about 12 %.

(*f*) The larval cuticle contains about 60 % of chitin, and the puparium about 47 %. The absolute amount of chitin remains unchanged during the formation of the puparium.

(*g*) The water-soluble fraction decreases from approximately 33 % to approximately 8 %.

(*h*) The alkali- or acid-soluble fraction increases from approximately 40 % to 53 %. The water-insoluble and alkali- or acid-soluble fraction increases from approximately 7 % to 45 %.

4. It is considered that almost all the non-chitinous substance in the larval cuticle and puparium is protein, which is comparatively water-soluble in the larval cuticle and water-insoluble in the puparium.

5. The water-soluble protein which is extracted from larval cuticles in the cold or on boiling, and from puparia by autoclaving, shows peculiar properties which distinguish it from other proteins. It is characterized by high solubility in hot water, and after being precipitated in trichloroacetic acid, it redissolves on heating. It gives an intense Millon reaction.

6. The hardening of the puparium can be explained in terms of the following facts:

(*a*) The higher degree of chitin orientation.

(*b*) The closer packing, by dehydration, of the substances which form the puparium.

(*c*) The increasing insolubility of the proteins in the puparium.

(*d*) The change from a dispersed phase of chitin in protein to a condensed chitin-protein association. A series of X-ray comparison photographs is given and these show that the chitin pattern is altered in the natural puparium. The degree of crystallinity apparently deteriorates along the *a* and *c* axes but not along the *b* axis. It is suggested that this is due to a mixed crystallization of chitin and protein.

(*e*) The loss of swelling power in the puparium.

7. It is not necessary to postulate the existence of special incrustations, and the term 'chitinization' hitherto often used for describing the process of hardening in the insect cuticle is misleading. There is no increase in chitin content, but the swelling power of the puparium resembles closely that of chitin itself.

APPENDIX (*added in proof*)

Since this paper was communicated, two papers by M. G. M. Pryor (1940) have appeared, which deal with the mechanism of the hardening process in the insect cuticle. While it is not possible to discuss this work in detail here, we should like to add the following remarks:

1. We have shown that the hardening process is intimately connected with the increasing insolubility of the protein constituent of the cuticle. Pryor's suggestion concerning the introduction of aromatic cross-linkages in the protein during this stage would help to explain the mechanism of the change; but for a complete understanding of the condition and function of the hardened cuticle it is necessary to consider also other factors which are associated with the process.

2. The identical values of the nitrogen content in the non-chitinous fraction of larval cuticles and puparia show that added phenolic compounds cannot be present in the puparium in other than very small amounts. This is the more so if the chromogen, as suggested by Pryor, does not contain nitrogen.

3. While we agree with the term 'sclerotized' protein, we consider that 'sclerotin' as a specific name for a protein is inappropriate. Any tanned or fixed protein, irrespective of its amino-acid composition, has as much claim to the term 'sclerotin' as these insect proteins.

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DESCRIPTION OF PLATES

Plate 1

FIGURE 5. X-ray photograph of larval cuticle, taken before drying. Beam parallel to the surface and parallel to the longitudinal axis. The transverse axis is oriented vertically.

FIGURE 6. X-ray photograph of white pupa cuticle taken before drying. The orientation of the specimen is the same as in figure 5.

FIGURE 7. X-ray photograph of puparium obtained from a larva which was ligatured. Beam perpendicular to the surface.

FIGURE 8. Same specimen as in figure 7, but with the beam directed parallel to the surface and parallel to the longitudinal axis. The transverse axis is oriented vertically.

FIGURE 9. X-ray photograph of dried larval cuticle. Beam perpendicular to the surface.

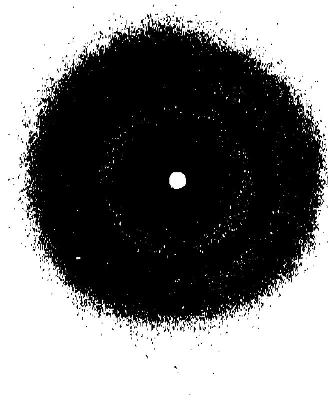
FIGURE 10. Same as in figure 9, but with the beam parallel to the surface and parallel to the longitudinal axis. The transverse axis is oriented vertically.

Plate 2

FIGURE 11. X-ray photograph of dried white pupa cuticle. Beam perpendicular to the surface. The transverse axis of the pupa body is oriented vertically.

FIGURE 12. White pupa cuticle. Beam parallel to the surface and parallel to the longitudinal axis. The transverse axis is oriented vertically.

FIGURE 13. White pupa cuticle. Beam parallel to the surface and parallel to the transverse axis. The longitudinal axis is oriented vertically.



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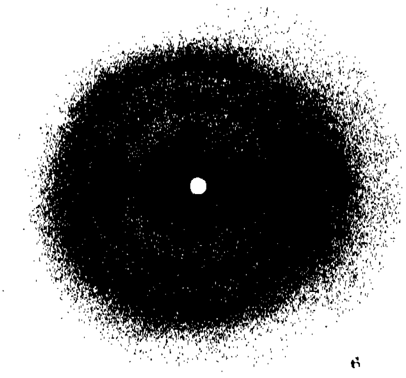

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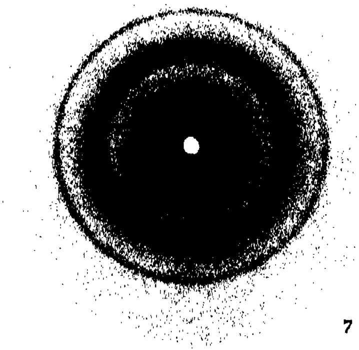

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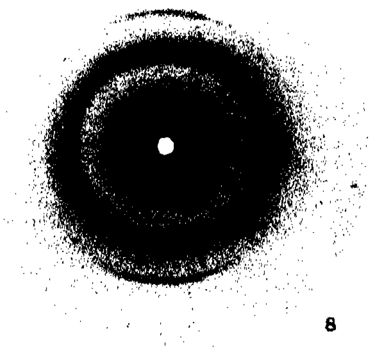

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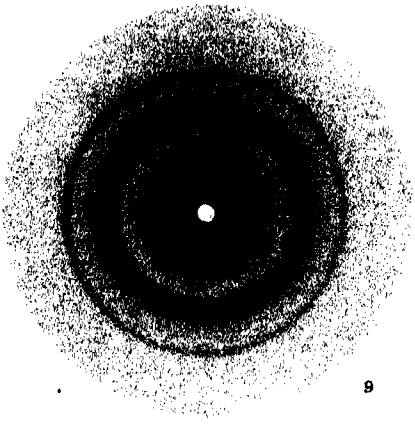

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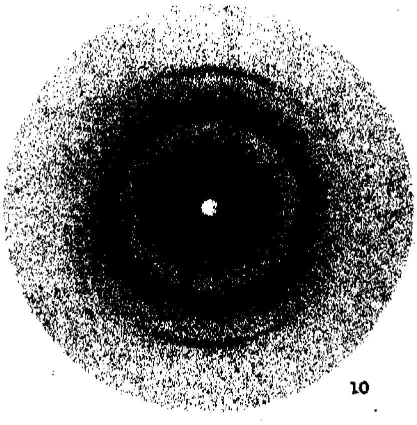

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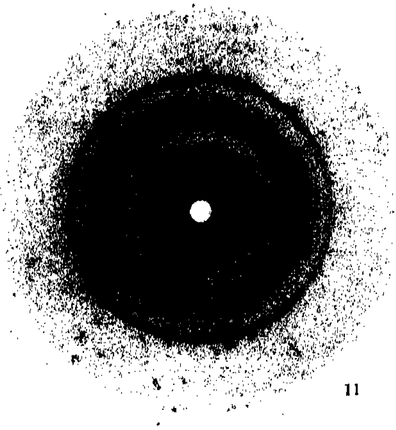
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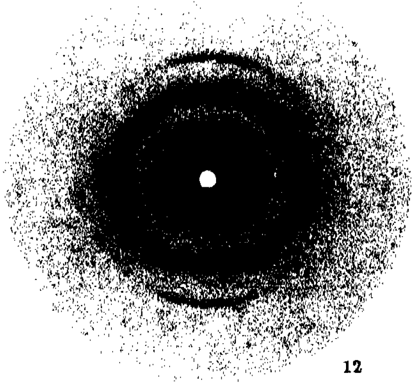
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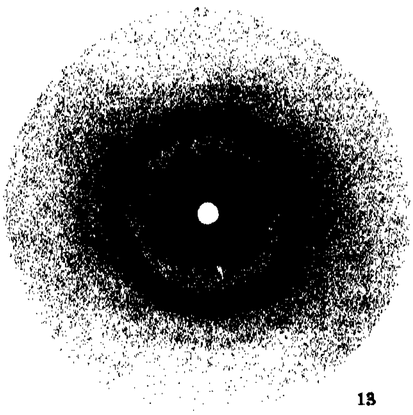
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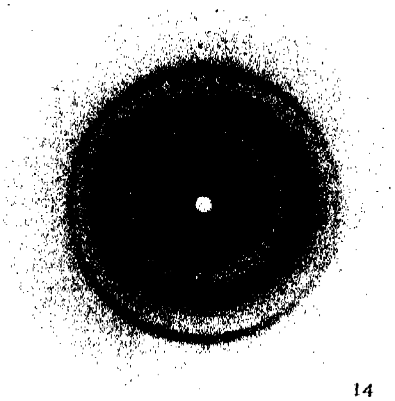
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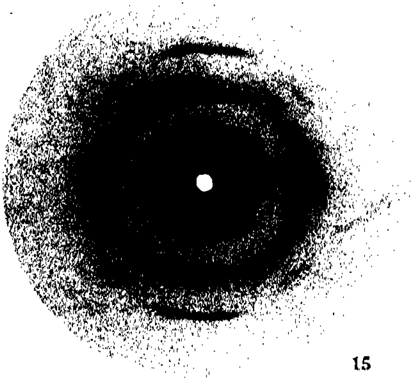
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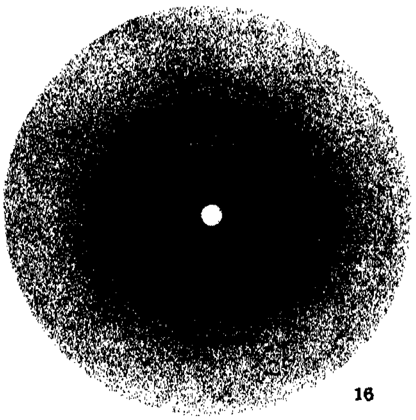
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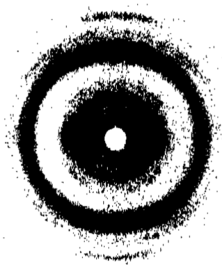

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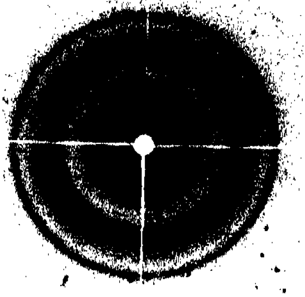

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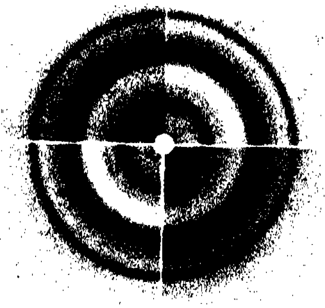

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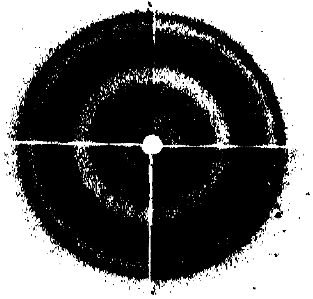

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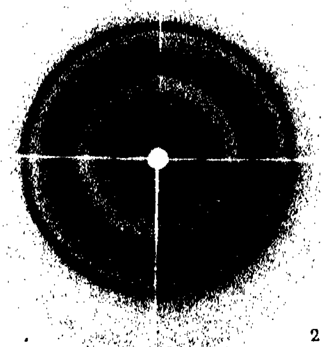

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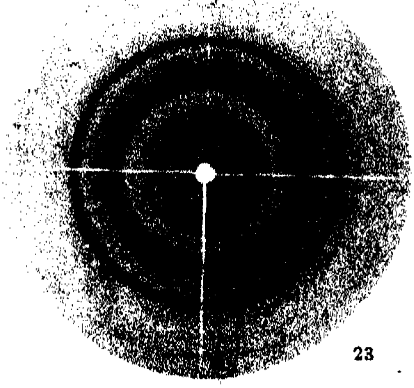

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FIGURE 14. X-ray photograph of the dry puparium. Beam perpendicular to the surface, the orientation being the same as in figure 11.

FIGURE 15. Puparium. Orientation is the same as in figure 12.

FIGURE 16. Puparium. Orientation is the same as in figure 13.

Plate 3

FIGURE 17. X-ray photograph of puparium which was purified in diaphanol. Beam parallel to the surface and parallel to the transverse axis. Same orientation as in figures 13 and 16.

FIGURE 19. Comparison photographs using powdered material. Top right and bottom left = white pupa cuticle. Top left and bottom right = larval cuticle.

FIGURE 20. Top right and bottom left = white pupa cuticle. Top left and bottom right = puparium.

FIGURE 21. Top right and bottom left = purified puparium mixed with protein. Top left and bottom right = natural puparium.

FIGURE 22. Top right and bottom left = purified white pupa cuticle mixed with protein. Top left and bottom right = natural white pupa cuticle.

FIGURE 23. Top right and bottom left = purified white pupa cuticle. Top left and bottom right = purified puparium.

New termite intercastes

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Intercastes of two species of termites are described, and their significance is discussed.

1. INTRODUCTION

The occurrence of intercastes in colonies of social insects is surprisingly infrequent. In almost all of them the abnormal individual is clearly a member of one caste and possesses only a few and usually unimportant features of another caste. Among the termites the most striking intercastes as yet recorded are some soldiers of the primitive family Kalotermitidae, which differ from normal soldiers only in the possession of rudimentary wings and in some cases in the abnormal development of the gonads. More numerous and varied intercastes have been found in colonies of ants, but few of them show combinations of many highly contrasting features.

An intercaste found by the writer in a colony of *Nasutitermes guayanae* (Holmgren) in Trinidad, British West Indies, in March 1937, appears to be entirely unique in the possession of so many important characters of two castes that it cannot readily be assigned to one or the other. The head is predominantly that of a nasute soldier, but it bears the compound eyes and ocelli of the reproductive caste, which predominates in the thorax and abdomen. By an odd coincidence, two other intercastes were found in June of the same year, by Miss Patricia Atteck, in an observation colony of *Microcerotermes arboreus* Emerson, which had been set up in the laboratory by the writer in December 1936. These are soldiers with rudimentary wings and with the vestigial compound eyes more highly developed than they are in a normal soldier. Both *Nasutitermes* and *Microcerotermes* belong to the highest family of termites, the Termitidae, in which only a very few intercastes, between workers and soldiers, have been recorded.

In view of the great interest of intercastes, especially in connexion with mystery of caste determination, a detailed description of these unique forms from Trinidad is given here. It is followed by a brief account of other known intercastes and a discussion of their relation to theories of caste determination.

2. THE INTERCASTE OF *NASUTITERMES GUAYANAE* (HOLMGREN)

(*Eutermes guayanae* Holmgren. *Jb. Hamburg. wiss. Anst.* 1911, **27** (Beih. 2), 254–256.)

General features

A single specimen of the intercaste was taken from a large nest, about 2 ft. in diameter, on a dead tree stump in a young grapefruit plantation, recently cleared of forest, at Spring Hill Estate, Tamana, not far from the centre of Trinidad, on 10 March 1937. A portion of the nest was brought to the laboratory, placed in a jar with chloroform, and the termites then collected. The intercaste was therefore dead when first seen and there was no opportunity to observe its behaviour.

All other termites taken from this colony, including alates, nymphs with wing buds, soldiers and workers, appear to be normal. No abnormal specimens have been found in the many other colonies of this species examined in Trinidad, where it is one of the common builders of arboreal nests.

In size the intercaste is intermediate between the soldier and the last reproductive nymph. All external features of the head, except the eyes and the antennae, are more or less soldier-like. The antennae resemble closely those of the imago, and the compound eyes and ocelli recall those of a reproductive nymph. The mouthparts are essentially soldier-like, but they approach those of the reproductive caste in the relatively large proportions of all parts, except the palps, in their larger and more numerous bristles and lighter colour. The pronotum is soldier-like in colour, but all parts of the thorax and abdomen, and their appendages, bristles and hairs resemble in form those of the last nymph of the reproductive caste. The pigmentation of the body, with the exception of the pronotum, approaches that of the imago, being darker than in a nymph and lighter than in a soldier. The distribution of characters is shown in Table 1.

TABLE 1. DISTRIBUTION OF CASTES REPRESENTED IN *NASUTITERMES* INTERCASTE

	Reproductive		Soldier
	Adult	Last nymph	(adult)
Head: form, colour, bristles, etc.	.	.	×
Compound eyes	.	×	.
Ocelli	.	×	.
Antennae	×	.	.
Labrum	.	.	×
Mandibles	.	.	×
1st maxillae: form, bristles, etc.	.	.	×
colour	×	.	.
Labium: form, bristles, etc.	.	.	×
colour, excluding gulamentum	×	.	.
colour, gulamentum	.	.	×
Thorax: form, bristles, etc.	.	×	.
colour, excluding pronotum	×	.	.
Pronotum: colour	.	.	×
Wings: excluding colour	.	×	.
Legs: form, etc.	.	×	.
colour	×	.	.
Abdomen: form	.	×	.
colour	×	.	.

The instar to which the intercaste belongs is a matter for conjecture. The head as a whole appears to be fully adult, being as maturely developed as that of a normal adult soldier. The antennae differ little from those of the imago, showing none of the peculiar features of a nymph, and the pigmentation of the body, though imperfect, is essentially adult. On the other hand, the form of most parts of the body and its appendages, and their bristles and hairs, are nearer to those of the last reproductive nymph. As a whole, the soldier-like characters may therefore be regarded as adult and those of the reproductive caste as immature.

The sex of the intercaste is clearly female, the seventh abdominal sternite being characteristically enlarged and the eighth and ninth greatly reduced.

TABLE 2. DIMENSIONS OF INTERCASTE AND NORMAL INDIVIDUALS OF *NASUTITERMES GUAYANAE* (HOLMGREN)

	Intercaste mm.	Soldier mm.	Reproductive nymph, last instar ♀ mm.	Alate ♀ mm.
Length, head and body	7·9	4·6	9·5	9·4
Head: length	2·02	1·85	1·78	1·78
width without eyes	1·44	1·13	1·36	1·42
Abdomen, length	3·96	—	5·79	5·12
Antenna, length	2·76	1·96	2·38	2·45
Labrum: length	0·16	0·15	0·47	0·55
width	0·35	0·25	0·56	0·56
Mandible, left, length	0·40	0·29	—	—
Maxilla: length without palps	0·75	0·53	1·00	1·38
length of palp	1·11	0·95	0·98	1·20
Labium: length without palps	1·05	0·65	1·11	1·17
length of palp	0·73	0·55	0·68	0·76
Compound eye, greatest diameter	0·25	—	0·44	0·51
Ocellus, diameter, transverse	0·13	—	0·05	0·13
Pronotum: length	0·58	0·24	0·80	0·78
width	1·04	0·58	1·31	1·36
Wing-bud, left anterior, length	2·77	—	3·82	—
Tibia of third legs, length	2·13	1·44	1·76	1·82

The normal individuals are typical specimens from the same colony as the intercaste.

Head capsule

The form of the head is soldier-like throughout but with some modification. It is larger (2·02 mm. long and 1·44 mm. wide) and more robust than that of a normal soldier (1·85 mm. long and 1·13 mm. wide), and the rostrum is shorter and thicker. The posterior ventral portion of the head is widened to accommodate the maxillae and labium, which are of much larger proportions than in the soldier.

The surface texture and minute sculpturing of the head appear to be identical with those of the soldier. The bristles on all parts of the head are exactly soldier-like, and the numerous fine hairs on the head of the imago are entirely lacking.

The colour of most of the head is soldier-like, but very slightly lighter throughout. On the posterior dorsal portion of the head, adjacent to the pronotum, is a lightly pigmented patch resembling a similar, but slightly smaller and darker, patch on the head of the imago. This is absent in the normal soldier. A light median streak on the top of the head of the intercaste, from the posterior margin to near the middle of the head, corresponds to a similar, but shorter and darker line, on the head of the imago. This streak is absent or very faint in the soldier.

The fontanel of the imago is not represented by any visible external structure on the top of the head of the intercaste. In the last nymph of the reproductive caste it is recognizable as a slight depression.

Eyes

The compound eyes, of which I have found no trace in any soldier of *N. guayanae*, occupy the same position as in the imago. They are less prominent and considerably smaller (0·25 mm. in greatest diameter) than in the imago (0·51 mm.) or even in the last reproductive nymph (0·44 mm.). They are more elliptical than in the imago, and their upper margin is slightly angular instead of evenly rounded. The facets are weakly defined, being separable with difficulty and only in parts of the eyes, and appear to be less highly developed than in a penultimate reproductive nymph. The general colour is pale violet-grey, with a paler narrow margin. A ring of pale yellow chitin surrounds the eye. In the imago the entire eye is very dark—black or nearly so—and in the last nymph of the reproductive caste it is almost fully pigmented, with only a slight tinge of violet. In the penultimate nymphal instar the eyes are paler than in the intercaste and less prominent, but the facets are more clearly defined. The compound eyes of the intercaste have thus a rudimentary appearance, and it seems unlikely that they were capable of function.

The ocelli are whitish, translucent areas resembling those of the imago in size and structure, as seen in surface view. They are, however, displaced and distorted by the nasute form of the head. They are on the sides of the rostrum, about three times their diameter from the compound eyes, against one-half in the imago. Each ocellus is abnormally prolonged towards the tip of the rostrum, and its margins are not sharply defined, being invaded here and there by small pigmented areas, especially towards the anterior pointed end. The ocelli are not prominent—in the imago they project conspicuously, but only very slightly in the last nymph.

Antennae

The antennae had suffered an injury, healed before capture, which amputated the right antennae at the sixth segment and, presumably at the same time, left an indentation on the sixth segment of the left side. The terminal segment of the left antenna, also, appears to be abnormally small, possibly by injury. The appearance of the healed surfaces suggests that these injuries occurred after the previous moult and therefore too late to affect the development of the insect.

The antennae of the intercaste resemble closely those of the imago in size, number of segments (sixteen, against fourteen in most soldiers), form of most segments, pigmentation, bristles and hairs. They seem to be fully mature, lacking all the immature features of the antennae of a nymph. They are slightly thicker, especially segments 4 and 5, than in the imago; segments 2–4 are longer than in the imago, though not so elongated as they are in the soldier. There are no well-defined features in common with the soldier, in which the antennae are considerably smaller, more slender and darker than in the imago and intercaste. The slight elongation of segments 2–4 of the intercaste is probably, but not certainly, to be regarded as a soldier-like character of minor importance.

Mouthparts

Labrum.

The labrum resembles that of the soldier but is considerably larger (0·16 mm. long and 0·35 mm. wide against 0·15 and 0·25) and pale reddish brown instead of dark reddish brown. It bears three long bristles, two near the distal margin and one—representing a pair of which the other is lacking—near the middle, and a few minute hairs. In the soldier there are usually two pairs of bristles similarly situated, but considerably smaller. In the imago the labrum is much larger (0·55 by 0·56 mm.) and paler than in the soldier and the intercaste, and it bears many long bristles and smaller hairs. The labrum of the intercaste is therefore intermediate in size, pigmentation and length of bristles between those of the soldier and the imago, but it appears to lack any structures peculiar to the reproductive caste. An interesting feature of the labrum of many of the Termitidae is the presence along its anterior margin of six specialized hairs, each set in a socket with a projecting rim; these are present in similar form in all castes of *N. gúayanae* and also in the intercaste.

Mandibles.

The mandibles are soldier-like, differing mainly in their larger size (0·40 mm. long and 0·34 mm. wide against 0·29 and 0·26). The distal part of each is reduced to the sharply pointed, deeply pigmented tooth which is characteristic of the nasute soldier. Along the inner margin of each mandible there is a heavily chitinized and deeply pigmented area, with a rough, ridged surface. This structure corresponds to a somewhat similar area on the posterior part of the inner margin of the mandibles of the imago and worker. In the soldier it is present as a minute rudiment, whereas in the intercaste it is developed to about the same relative degree as in the imago. The pigmentation of the basal part of the mandibles of the intercaste is somewhat lighter than in the soldier.

Maxillae.

The principal features distinguishing the maxillae of the soldier of *N. guayanae* from those of other castes are the small size of all parts except the palps, which are relatively long, and the reduction in size and number of the bristles and hairs. In these features the intercaste is intermediate between imago and soldier, but nearer to the soldier. The maxillae as a whole are relatively larger than in the soldier, and the bristles considerably larger, especially in the row along the inner side of the lacinia. Both soldier and intercaste lack the complex, brush-like series of hairs on the tip of the galea of the reproductive and worker castes. These hairs are presumably sensory, and apparently not required by the soldier.

Labium.

The labium of the intercaste, like the maxillae, is intermediate between those of the imago and the soldier, but nearer to the latter. The palps are relatively long, as in the soldier, but the other parts are considerably larger than in the soldier. The gulamentum is even wider than in the imago, but resembles that of the soldier in its heavy chitinization and the relatively small number and size of the bristles.

Thorax

The general features of the thorax are essentially those of the reproductive caste. The greatest abnormalities are in the wings and the pronotum, which are described in detail below, and in the modification of the dorsal sclerites of the meso- and metathorax associated with the wings. The thoracic structures seen in lateral and ventral view resemble closely those of the last reproductive nymph, being smaller in proportions and less heavily chitinized than those of the imago. The bristles and hairs are about the same in number, size and distribution as in the nymph, being smaller and much less numerous than in the imago. The pigmentation, however, resembles that of the imago, though paler throughout and almost lacking the light reddish tinge of the latter. The colour of the pigmented areas varies from very dark brown at the margins of a few of the sclerites through dull brown, with only a slight reddish tinge, to pale yellow-brown. The dorsal portions of the meso- and metathorax are dark reddish brown along the posterior margins of the meso- and metanota, fading anteriorly to pale dull reddish brown. A median white streak, rather wider than in the imago, extends along the meso- and metanota.

Pronotum.

The pronotum in general is similar to that of the reproductive caste but resembles that of the soldier in pigmentation. In form it resembles closely that of the last reproductive nymph, though slightly smaller (0·58 mm. long by 1·04 mm. wide against 0·80 and 1·31). It is much smaller than the pronotum of the imago (0·78 by 1·36) and much larger than that of the soldier (0·24 by 0·58). Most of it is dark brown, deepening towards the anterior margin and fading to light reddish brown over most of the central portion and at the middle of the posterior margin. A continuous pale yellow median line traverses the pronotum; this is faintly represented in both imago and soldier. A pale yellow spot near the antero-lateral margins is also feebly present in imago and soldier. In general, the pigmentation is much more like that of the soldier (dark reddish brown) than of the imago (light reddish brown).

The projecting anterior margin, which gives the characteristic saddle-shaped appearance to the pronotum of the sterile castes of Termitidae, is as feebly represented in the intercaste as it is in the imago and reproductive nymph. The bristles on the pronotum of the intercaste, though rather fewer in number, resemble in position and size those of the reproductive nymph; in the imago they are much more numerous and in the soldier much less so. The numerous small hairs on the pronotum of the imago are lacking in the reproductive nymph, intercaste and soldier.

Wings.

The wings are somewhat similar to those of the last nymph of the reproductive caste, but differ in almost all details, in some of which they appear to be at a later stage of development. They are of nearly the same relative length as in the nymph, scarcely half as wide and much thinner, though much thicker than in the imago. Their inner margins are fused with the meso- and metanota, but they do not meet in the middle, leaving much of the meso- and metanota exposed. In the basal sclerites of the forewings (a dissection to expose this part of the hind-wings was not made) the scale and humeral suture are clearly though faintly discernible. These structures are not recognizable in the nymph.

The wing veins, bristles and hairs are developed to about the same extent as in the nymph. The colour of the wings is dull dark brown basally, fading to pale, dull yellow distally and along the inner margins. They are nearly opaque. In the wings of the nymph there is scarcely any dark pigment.

The above combination of characters shows that, though probably representative of the same instar as the last reproductive nymph, the wings of the intercaste may perhaps be imperfect adult rather than juvenile structures.

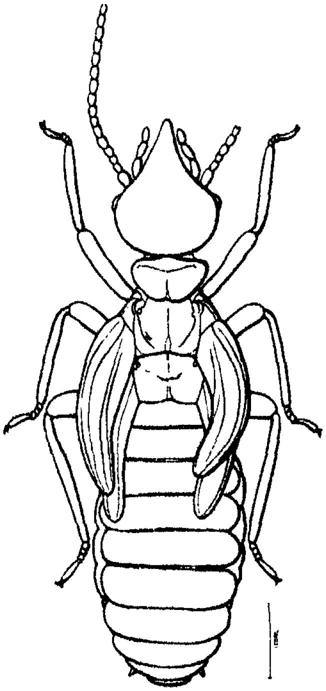

FIGURE 1. *Nasutitermes guayanae* (Holmgr.) intercaste.

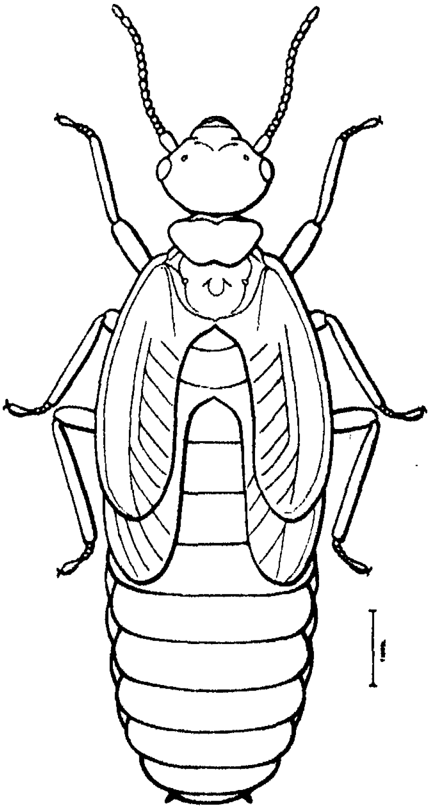

FIGURE 2. *Nasutitermes guayanae.* Normal nymph, last instar, ♀.

Legs.

In form the legs resemble fairly closely those of the last reproductive nymph, being smaller and less robust, and having fewer bristles and hairs, than those of the imago. Their general colour is very pale, dull yellow, with

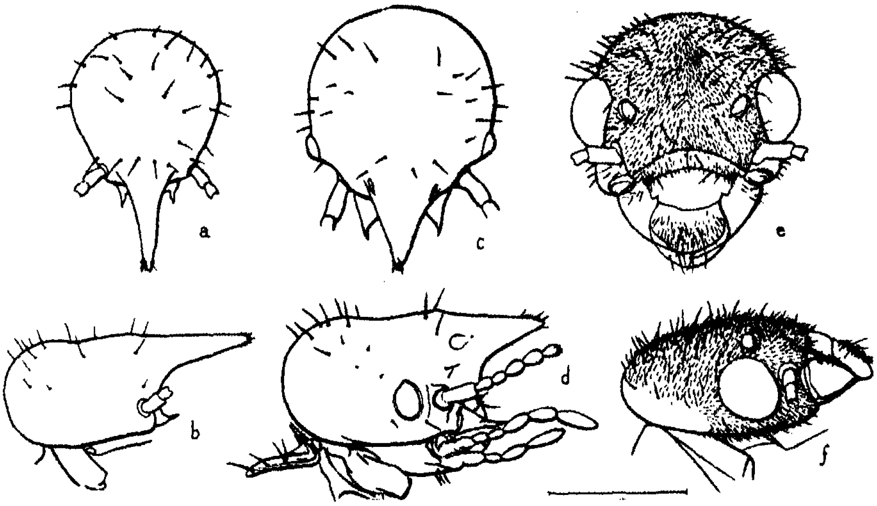

FIGURE 3. *Nasutitermes guayanae.* Soldier *a–b*, intercaste *c–d*, alate ♀ *e–f*.

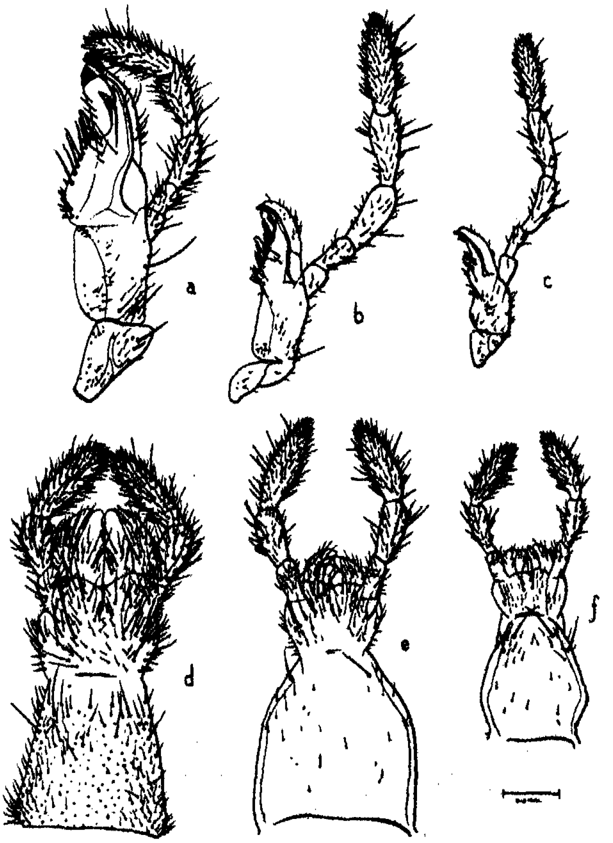

FIGURE 4. *Nasutitermes guayanae.* Maxilla, left, ventral view, of alate ♀ *a*, intercaste *b*, soldier *c*. Labium, ventral view, of alate ♀ *d*, intercaste *e*, soldier *f*.

light reddish brown markings on the coxae and along the femora. This distribution of pigment is somewhat similar to that of the imago, but the bright reddish yellow tinge throughout the legs of the imago is almost entirely lacking. No soldier-like features are apparent in the legs of the intercaste.

Abdomen

The external features of the abdomen resemble closely those of a female of the reproductive caste in the last nymphal stage, differing mainly in smaller size, darker pigmentation, and less numerous bristles and hairs. The dense brown pubescence of the abdominal pleura of the female imago are lacking in both intercaste and nymph. The colour of the tergites and sternites is reddish brown, almost exactly as in the imago. The pleura are dull white as in the nymph.

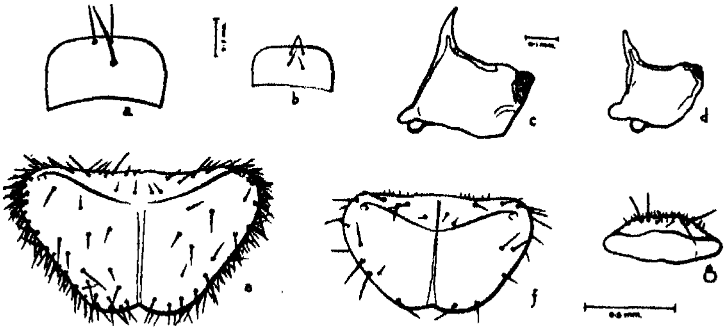

FIGURE 5. *Nasutitermes guayanae*. Labrum of intercaste *a*, soldier *b*. Mandible, left, dorsal view, of intercaste *c*, soldier *d*. Pronotum of alate ♀ *e*, intercaste *f*, soldier *g*.

3. The intercaste of *Microcerotermes arboreus* Emerson

(*Microcerotermes arboreus* Emerson. *Zoologica, New York*, 1925, 6, 453–455.)

The two abnormal soldiers representing the new intercaste were found in June 1937, in an observation colony at the Imperial College of Tropical Agriculture, by Miss Patricia Atteck, Technical Assistant in the Entomology Department. The colony was established by the writer in December 1936, by placing a portion of an arboreal nest, about 10 cm. in diameter, on a plate of glass about 40 cm. square, surrounded by a moat filled with water. A small piece of a dead branch of a tree was placed on the glass, and a layer of earth, about 3 mm. thick, covered by a small glass plate and a piece of black paper to exclude light. The soil was kept moist by a wick of blotting paper dipping in a tube of water. In such conditions many kinds of termites can be maintained indefinitely. They soon build runways leading to sources of food, soil and water, and the only obstacle to observation in the narrow space between the glass plates is the termites' habit of obscuring the glass with faecal matter. It is hoped that a solution of this difficulty may be found, making it possible to observe many phases in the life of a termite colony, including that of the king and queen, in almost natural conditions.

With the intercastes, a series of alates, two soldiers, and many workers and nymphs from the same colony were preserved. These appear to be entirely normal, except for the lack of the anterior median lobe on the pronotum of one of the soldiers; this lobe is absent also in one of the intercastes. It is possible, if not probable, that the abnormality of the intercastes was brought about by the unnatural conditions of a small colony in the laboratory. Unfortunately the colony died out a few months after the intercastes were found and the presence or absence of a functional king and queen, at that time, was not ascertained.

Table 3. Dimensions of intercaste and normal individuals of *Microcerotermes arboreus* Emerson

	Intercaste mm.	Soldier mm.	Reproductive nymph, last instar mm.
Length, head and body, without mandibles	4·9	4·2	6·8
Head: length without mandibles	1·76	1·80	—
width	0·89	0·95	—
Mandible, right, length	0·91	1·04	—
Rudiment of eye, diameter	0·15	0·07	—
Pronotum: length	0·44	0·33	0·53
width	0·64	0·56	0·73
Wing buds, anterior, length	1·42	—	2·58
Tibia of third legs, length	0·87	0·69	0·84

The normal soldier is from the same colony as the intercaste. The reproductive nymph is a typical specimen from another colony.

The two specimens of the intercaste are identical in all significant external characters, except that in one of them the pronotum is slightly smaller and lacks the anterior lobe, and that in the same specimen the wing scales and humeral sutures are less developed than in the other.

The abnormal characters of the intercastes are in the vestigial compound eyes, the form of the pronotum, and the wing-buds. No other external features of significance appear to separate them from normal soldiers.

In most normal soldiers of *Microcerotermes arboreus* in Trinidad the compound eye is represented by a small pigmented area behind the antenna. The pigment varies from light yellow, only slightly darker than the pale yellow of the head generally, to dark brown. No clear indication of facets has been seen. In the intercastes the pigmented area has a diameter of about 0·15 mm., about twice as great as in any normal soldier. The eye is bounded on its anterior margin by a semicircular rim of dark reddish brown chitin. Along this semicircle is a row of about eight facets, and about thirty are distributed over the middle and posterior parts of the eye. The facets are more or less circular and about half the size of those of the imago. Their colour varies from almost white to dark brown.

The pronotum of each intercaste is somewhat similar in size and form to that of a reproductive nymph in its last instar, being larger, wider and flatter than in the soldier. The ridge on the anterior margin, which in the sterile castes of the Termitidae gives the pronotum a characteristic saddle-like form, is developed in one of the intercastes to about the same extent as in the reproductive nymph. In the other intercaste this ridge is absent, its place being occupied by a slight depression. One soldier in the same colony, though otherwise normal, has a pronotum of this form.

The colour of the pronotum of the intercastes is pale, translucent reddish yellow along the margins, fading towards the middle, where pigment is almost lacking. In one of them the raised anterior margin of the pronotum is slightly darker than the other parts. The bristles and hairs on the pronotum of both intercastes are similar to those of a reproductive nymph, being slightly fewer and smaller than in the imago but larger and more numerous than in the normal soldier.

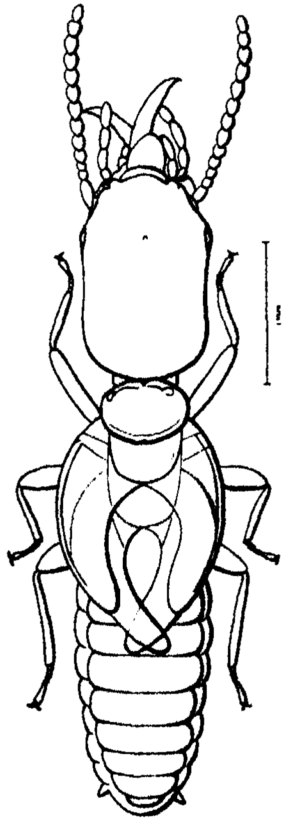

FIGURE 6. *Microcerotermes arboreus* Emerson, intercaste.

The wing buds bear a general resemblance to those of a reproductive nymph before its final moult, but differ in all details. They are shorter, narrower and thinner than in the reproductive nymph, and the chitinization of the basal structures and of the veins is of greater degree. The differentiation of the basal scale and humeral suture is faintly but unmistakably discernible. The wings are translucent, with little pigment, of a pale yellow colour. The wing buds of a normal nymph are without pigment. The wings of the intercastes of *Nasutitermes* and of *Microcerotermes* are similar in degree of development.

All parts of the abdomen, including the terminal sternites, of the intercastes of *Microcerotermes* appear to be exactly similar to those of a normal soldier.

4. OTHER KNOWN INTERCASTES OF TERMITES

In the literature I have found records of only two principal types of termite intercastes: soldiers with wing buds in a few species of the primitive family Kalotermitidae, and forms combining features of the soldier and worker castes in *Reticulitermes*, of the family Rhinotermitidae, and in *Nasutitermes*, of the Termitidae. Emerson (1926) writes: 'Soldiers are often found with wing buds which may be pigmented in the Kalotermitidae. None with wing buds have ever been found in the higher termites, however', and 'No thoroughly verified case of fertile workers or workers with wing buds has ever been described'. In the innumerable termite colonies which he has studied, Emerson (1935) has reported only one with soldier-worker intercastes. Snyder (1933) similarly emphasizes the rarity of termite intercastes, and he questions Heath's (1928) observation that certain soldiers of *Zootermopsis nevadensis* (Hagen) laid fertile eggs. Castle (1934), however, writes that he obtained soldiers of *Z. angusticollis* with well-developed testes and ovaries, both in the field and in isolated groups in the laboratory. These he regards as supplementary reproductives. They were without trace of wings.

In *Kalotermes occidentalis* Walker, recorded from Arizona and western Mexico, *all* soldiers according to Snyder (1933) have wing buds or traces of wings. A character abnormal in other termites appears to be normal in this species, as are the vestigial eyes of soldiers of the Kalotermitidae and even of the Termitidae.

In the common North American subterranean *Reticulitermes tibialis* Banks, of the family Rhinotermitidae, Snyder (1933) has described 'a male half worker, half soldier form'. The head is coloured as in the soldier, and slightly longer than in the worker; the mandibles and labrum are 'typically worker-like, but are extended as in the soldier'. Snyder was unable to decide whether this termite is merely 'a worker of abnormal development' or a true intercaste.

In *Nasutitermes* (*N.*) *myersi* Snyder, from Brazil, Snyder (1933) recorded an individual with the colour of a soldier, a short rostrum, but with worker-like mandibles and in general features more like a worker than a soldier.

Emerson (1935) found fourteen soldier-worker intercastes in a single colony of *Nasutitermes* (*N.*) *similis* Emerson—a species very close to *N. guayanae*—at Kartabo, British Guiana, in 1920. 'The intercastes are more soldier-like than worker-like, but most of them deviate from the soldier in having a shorter head projection and an extra tooth on each mandible.' Emerson's comments on the significance of this exceptionally interesting discovery are mentioned below.

The intercastes of ants are described in papers by Wheeler (1928*b*, 1937).

5. ORIGIN OF INTERCASTES AND THEORIES OF CASTE DETERMINATION

The greatest interest of intercastes lies in their relation to the problem of caste determination and any explanation of their origin must depend largely on an understanding of caste determination in general.

Unfortunately the mechanism of caste determination in termites and ants remains almost completely unknown. The evidence available up to a few years ago, and the theories based upon it, have been reviewed by such writers as Wheeler (1928*a*), Imms (1919), Emerson (1926, 1939) and Castle (1934). The theories of 'intrinsic' or genetic determination, prior to hatching, have received little or no direct support from observations on termites. Thompson (1917) claimed that newly hatched nymphs of many genera of termites could be separated into two types, reproductive and soldier-or-worker, according to the size of the brain and gonads, but Hare (1934) has re-examined the material on which her conclusions were based and shown them to be without foundation. Bugnion's (1914) figure of a newly hatched nymph of '*Eutermes lacustris*' Bugnion with a 'corne frontale' (rostrum) is undoubtedly inaccurate, as Thompson (1919) pointed out long ago. The so-called rostrum is merely the labrum, which is disproportionately large in all first instar nymphs of *Nasutitermes* that I have examined. The rostrum of dwarf soldier nymphs, less than 2 mm. long, which I have found in very young colonies of *N. costalis* (Holmgren) in Trinidad, bears no resemblance to the distorted labrum in Bugnion's figure.

Great advances in the study of caste determination in termites are being made by Prof. S. F. Light and his associates at the University of California in Berkeley, using the primitive and adaptable *Zootermopsis angusticollis*. Some of these results, described by Castle (1934), show that the caste assumed by nymphs may be altered by the conditions in which they develop. Removal of the first soldiers to appear in a young colony causes an abnormally high proportion of nymphs to become soldiers. Extracts from the bodies of kings and queens, by alcohol or ether, inhibit the development of the reproductive organs in isolated nymphs. The inhibiting agent or agents are presumably excreted by the normal functioning reproductive termites, and their discovery lends striking support to Wheeler's concept of 'trophyllaxis' as a directing influence in the constitution and development of insect societies. If the mechanism of caste determination as thus far elucidated in *Zootermopsis* is the same in all termites, it would appear that caste is determined after hatching by certain activities, as yet little known, of the termites.

In many of the bees and wasps it has long been known or at least suspected that the caste assumed by females depends on the quality or quantity of food supplied to the larvae. In ants the more complicated problem of caste determination is perhaps even farther from solution than in the termites. Wheeler (1937), in the last work completed before his death, discussed the available evidence, including the known intercastes and other anomalies. The most striking of these were found in Trinidad by Dr Neal Weber, in a remarkable colony of a fungus-growing ant, *Acromyrmex octospinosus* Reich, in May 1935, about a year before the discovery of the termite intercastes described here. No less than 164 anomalous individuals were taken from this nest. Wheeler regarded some of them as the first known 'mosaics' between the female reproductive and the worker caste, and believed that they prove the correctness of the 'blastogenic' theory of caste determination in ants. He failed, however, to show that no alternative explanation of these anomalous forms was possible, and Whiting (1938) has recently shown that they may be intersexes or intercastes, and not mosaics in the genetic sense in which the term is usually applied. Whiting inclines towards the trophogenic theory of caste determination. An answer to the problem remains a subject for future investigation.

The above discussion throws little light on the origin of any known termite intercastes, and no adequate explanation of their occurrence has been advanced. Emerson (1935) writes regarding his soldier-worker intercastes of *Nasutitermes similis*: 'whether the effect is due to some unknown environmental influence or due to a genetic influence upon development cannot be determined from the available data'. There are, however, a few points worth discussing here.

The extreme rarity of termite intercastes suggests at first sight that they are not caused solely by abnormal conditions operating after hatching. Such factors as quantity and quality of food, including secretions of other members of the colony, injury, isolation, and so forth, must vary so much in nature that abnormalities, if caused by variation in these factors, would probably have been found more frequently by collectors of termites. On the other hand, if they are produced as a result of disturbances within the chromosomes, prior to hatching, the rarity of intercastes would be not only explainable, but to be expected. The above considerations could be extended to caste determination in general, in support of genetic determination.

It is obvious, however, that the rarity of intercastes is not altogether incompatible with 'extrinsic' theories of determination like that of Castle. It is possible that all nymphs hatch with equal potentialities for all castes, and that when subsequent conditions bring into operation the factors for one caste, the factors for other castes are thereby rendered completely ineffective. The condition of an individual in which factors peculiar to more than one caste are in operation may be so unstable that its persistence is extremely rare. An intercaste like that of *N. guayanae* described here may be the result of the fortuitous and rare coincidence of several factors.

In the case of the intercastes of *Microcerotermes arboreus* described here, it seems not unlikely that the abnormality was due to unnatural conditions in the laboratory. The presence or absence of a royal pair was not ascertained when the intercastes were found, but it is probable that no normal reproductives were present, and the development of features of the reproductive caste may be comparable to the abnormal development of the gonads in nymphs of *Zootermopsis* isolated from the king and queen. This explanation is supported to at least a slight extent by the fact that two intercastes of *Microcerotermes* were found. It is noteworthy in this connexion that Emerson's (1935) soldier-worker intercastes of *Nasutitermes similis* numbered no less than fourteen in one colony. This appears to be against the possibility of a chance disturbance in genetic constitution of these intercastes, unless, of course, it were one that concerned one or both parents, instead of their offspring individually.

It is of interest to note that the few known termite intercastes are between reproductive and soldier or soldier and worker castes, and that no forms combining characters of the reproductive and worker castes are known. This evidence, so far as it goes, supports the view of Emerson and others that the worker caste has been derived phylogenetically from the soldier, and not directly from the reproductive forms.

I am greatly indebted to Miss Patricia Atteck, Technical Assistant in Entomology at the Imperial College of Tropical Agriculture, for discovering *the intercastes of Microcerotermes, and also for making the drawings for* this paper. All of the intercastes have been examined by Dr A. E. Emerson, of the University of Chicago, and I am very grateful to him for his advice regarding them. Both he and Dr A. D. Imms have been kind enough to read the manuscript.

6. SUMMARY

An individual of *Nasutitermes* (*Nasutitermes*) *guayanae* (Holmgren) in the Termitidae, representing the only known termite intercaste combining intimately many important features of two castes, was found in Trinidad, B.W.I., in March 1937. The head is soldier-like in most features, but has compound eyes, ocelli and some other characters of the reproductive caste. The pronotum is intermediate between that of the soldier and reproductive castes; the wing buds are somewhat like those of a reproductive nymph, except that the pigmentation approaches that of an imago.

Two soldiers of *Microcerotermes arboreus* Emerson, also in the Termitidae, with wing buds, abnormally large vestigial eyes, and with the pronotum intermediate between those of the soldier and reproductive castes, were found in an observation colony, in Trinidad, in June 1937.

Previously reported termite intercastes appear to be merely soldiers with wing buds in a few species of the primitive Kalotermitidae and worker-soldier intercastes in *Reticulitermes* (Rhinotermitidae) and *Nasutitermes similis* Emerson and *N. myersi* Snyder (Termitidae).

The significance of termite intercastes is discussed. They support the view of Emerson and others that the worker caste arose phylogenetically from the soldier. An explanation of the origin of the intercastes, and their relation to theories of caste determination, must await further advances in the study of the latter problem.

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The structure of the cell wall in some species of the filamentous green alga Cladophora

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[Plate 4]

With a view to extending the work on *Valonia*, the cell walls of several species of *Cladophora* have been examined in detail by means of X-ray diffraction photographs and the microscope.

The walls are found to consist of layers in which the cellulose chains in any one layer are inclined to those in the preceding and subsequent layers at an angle whose average is less than 90°. The two sets of striations on the layers of the wall correspond closely to the directions of the cellulose chains.

Each set of chains forms a spiral round the cell, and the spirals are of opposite sign. One tends to be flat and the other steep.

On the whole, the steep spiral tends to become steeper on passing from the base of the filament to the tip, and the flat spiral flatter. In any one cell of the filament, the steep spiral is steepest at the end nearer the filament tip and the flat spiral flattest.

Wherever such changes in inclination occur, the angle between the chains tends to remain constant.

It is suggested that cell elongation is the factor causing the inclination of the steeper spiral to vary, and that the behaviour of the flatter spiral is best explained by the assumption of a protoplasmic mechanism causing a periodic change in the direction of cellulose chains through a constant angle.

The development of a branch cell is reviewed and is found to proceed as the above suggestions would indicate.

INTRODUCTION

The cellulose component of the plant cell wall is now well known to consist of long chains of β-glucose residues (Sponsler 1925–6; Meyer and Mark 1930) bound into ill-defined bundles or micelles (Hengstenberg and Mark 1928; Frey 1926), these in turn being further aggregated into fibrils which become visible on swelling the wall with certain reagents. The molecular chains of cellulose are clearly recognized as having definite orientation with respect to the axes of the cell, usually spiralling round the cell as in cotton (Frey 1926; Balls 1923) and some wood elements (Frey-Wyssling

1930; Preston 1934), sometimes lying almost parallel to the length of an elongated cell as in ramie (pericyclic fibres of *Boehemeria nivea*), and sometimes having both types of orientation in the same wall as in *Valonia*.

No clear evidence is yet available concerning the details of wall structure at the tips of elongated cells whose walls show spiral structure. Certainly in the case of *Valonia* the spiral obviously closes in towards two poles located in regions which may be termed the base and tip of the vesicles, but it is as yet hardly safe to generalize this observation to cover cells of an entirely different type. Nor has any real advance been made in the problems of wall deposition. The early accepted view that such deposition is merely a pseudo-crystallization controlled by the layers already present would seem to be inadequate in view of our knowledge of *Valonia* (Preston & Astbury 1937). Here we have a wall consisting of many layers, odd layers being built up of cellulose chains lying in one direction and even layers of chains making an angle of some 80° with the first set. Recent evidence points to the existence of a somewhat similar state of affairs in many of the cells of the higher plant (Freudenberg and Durr 1932; Frey-Wyssling 1935; Bailey and Kerr 1935; Preston 1939 *a*), but much of this evidence is unsatisfactory and in some cases is definitely contradicted by X-ray examination (Preston 1934, 1939 *b*; Kundu and Preston 1940). In *Valonia*, however, and in some cells of the higher plant where a similar state of affairs probably does obtain (sclerenchyma of *Vinca*, parenchyma of *Dahlia* tubers, some vessels (Preston 1939 *a*)), we have a definite rhythm of some kind, cellulose chains being deposited in each direction alternately. The present widespread belief in the universality of this phenomenon has led to several suggestions as to the mechanism involved (van Iterson 1936, 1937; Castle 1937) which will be discussed later in this paper.

In attempting to establish a geometrical connexion between the structure of the wall of a green alga such as *Valonia* and that of the higher plant, and at the same time obtain further evidence concerning the phenomena involved in wall deposition and growth, we are led first to examine those filamentous algae closely related to *Valonia*. Of the possible forms available, *Cladophora* was chosen for detailed study since the wall structure of its filaments can be ascertained with certainty, its method of growth has already been investigated in some detail, its growth form is intermediate between that of *Valonia* and the fibres of the higher plant, and its cells are not inconveniently small. The wall is thick and markedly stratified, and two layers (in the sense of Strasburger)* are readily visible, each consisting of many lamellae. Superficial examination indicates that the outer layer (*O*, figure 1 A) completely sheathes the whole filament and can clearly be seen uniting the two cells on each side of a cross wall (figure 1). The true nature of these walls is probably as represented in figure 1 B, although the multiplicity of lamellae in older cells obscures the real significance of the layers. The innermost wall (*i*, figure 1) envelopes only a single cell, each individual cell of a filament possessing such an inner wall peculiar to itself. The articulated appearance thus revealed leads immediately to the idea that with few exceptions (as, for instance, quoted by Brand (1901), and observed during the present work in *Cladophora arcta*) each cell has resulted from one division only. Such apical growth and division are clearly recognized in the literature, and have been further demonstrated in the course of the present work.

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* i.e. the layers are a coarse feature and are easily distinguished; each is a complex of lamellae visible under high magnification.

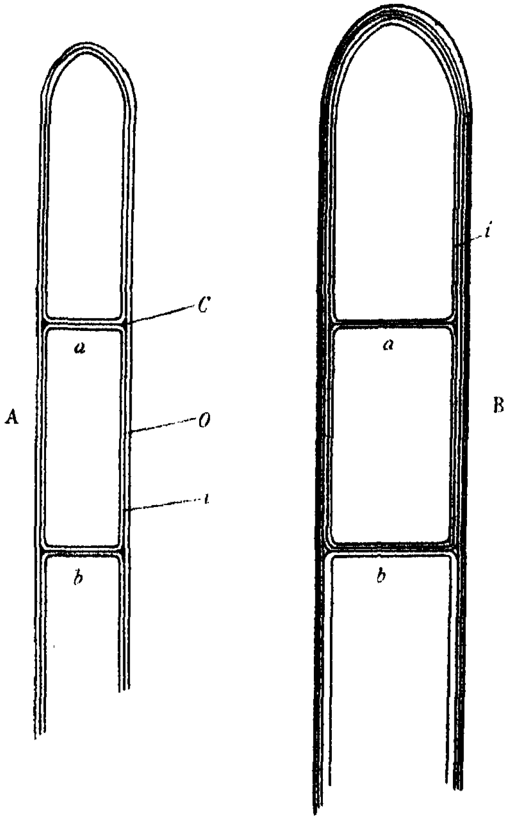


FIGURE 1

MATERIALS AND METHODS

Three species, all marine, were available for the present investigation—*Cladophora prolifera*, *Cl. arcta*, and *Cl. gracilis*. The first was obtained from the Stazione Zoologica at Naples, Italy, and the others were collected at Robin Hood's Bay and Filey, Yorkshire, England. Many of the laboratory observations were checked on living material, immediately after collection, in the research station at Robin Hood's Bay. In such considerations of wall structure as the present it is of fundamental importance that the experimental material should be as nearly as possible in the same condition as intact plants in their native habitat, and observations of preserved material were carefully checked, whenever possible, on living specimens. All three species were kept alive in the laboratory for several months in circulated, aerated sea water contained in glass tanks, so that living material was available continuously. The bulk of the determinations were made on the plants immediately after their introduction to the laboratory. Even after five months, however, the condition of the cultured material was not markedly changed from that of the original samples, and the plants were, in fact, actively growing.

X-ray determinations were made on dried samples which were treated with N/20 HCl to remove incrustations, washed in running water, and dried again. Each specimen consisted generally of a bundle of filaments as nearly parallel as possible taken from an appropriate part of the plant. No further treatment was necessary, for such cell inclusions as are found give no obvious diffraction images.

For observation under the microscope, however, other methods must be employed. The closely packed chloroplasts of the living filament makes impossible detailed observation of the wall, and while plasmolysis suffices in some cases to clear those parts of a filament near a cross wall, much more drastic processes are necessary for the bulk of the tissue. Most fixing reagents cause considerable swelling of the wall, with a consequent disturbance in wall structure. 70 % alcohol was found to be reasonably good, but the best results were obtained with picric acid fixatives. Specimens were therefore fixed both in alcohol and in a mixture of equal parts of absolute alcohol and a ⅛th saturated solution of picric acid in sea water. Fresh material was immersed in this latter solution for 2–3 hr. and then washed in running water. A brown granular deposit in the cells was removed by immersion in sodium hypochlorite solution (1 % available chlorine) for several hours. Immediately after this treatment, the wall was quite unswollen and the cell contents were sufficiently transparent to allow ready observation of details of wall structure. Too prolonged immersion in any of these reagents caused slight, progressive swelling so that critical determinations could be made only within 24 hours after treatment. Simple drying, followed by wetting out in distilled water, was also found to be a suitable procedure, though here again treatment with hypochlorite was *essential. Observation near cross walls on this material could be checked by plasmolysis of living material, and while in general no such check could be made for the rest of the wall surface, occasional degenerated cells (free of contents) in an otherwise fresh and living filament lead us to expect no* marked disturbance of wall structure on preserving.

The three species used are easily differentiated by morphological differences. In *Cladophora prolifera* the thallus is freely branched with frequently a single internodal cell, the branches themselves consisting of no more than two cells. In *Cl. arcta* branching is less frequent in the sense that many cells occur between one node and the next, and the branches contain from one to ten cells or more. *Cl. gracilis* is characterized by well marked secund branching, and the cells in both these latter species are much smaller than are those of *Cl. prolifera.*

MICROCHEMISTRY

Present knowledge of the chemical composition of the wall of *Cladophora*, in common with the bulk of the Chlorophyceae, leaves much to be desired. A complete treatment of this aspect of wall structure lies outside the scope of the present paper, but such observations as have been made during the course of the work may be of interest. The wall is definitely cellulosic in nature, although positive staining reaction for cellulose is often obtained only with difficulty, as already mentioned by Brand (1908). The wall swells readily in 70 % sulphuric acid, only slightly with zinc chloride, and not at all with Schweizer's reagent acting on the untreated wall. Only after the (fairly drastic) treatment necessary to obtain the chitin test (see below) does the wall swell, and finally dissolve, in this last reagent. It is particularly interesting that, although fresh material thus fails to swell in Schweizer's reagent, cellulose is completely removed (p. 61). This somewhat anomalous behaviour may perhaps be due to the presence of some non-swelling component in the wall, ensheathing the micelles. The difficulty of obtaining staining reactions for cellulose is in harmony with this view, which also recalls the condition in the higher plant as interpreted by some workers (e.g. the connexion between cellulose and lignin (Freudenberg and Durr 1932; Bailey and Kerr 1935; Frey-Wyssling 1935) and the *Fremdhaut-*

system of Ludtke (1932)). Swelling reagents would then cause high internal pressures which would force dissolved substances through minute fissures in the non-swelling layers. No swelling as a whole would be visible.

The nature of this non-swelling component is rather obscure. According to Mirande considerable quantities of pectin are to be found, a property which is apparently rather common among the Siphonales (Fritsch 1935), and callose is often present. Perhaps the most striking observations are those of Tiffany (1924) and Wurdach (1923), who describe the outer layers of the wall as chitinous in nature in spite of the earlier failure of von Wettstein to detect chitin. The presence of this substance in an otherwise cellulosic wall, which has been abundantly verified during the course of the present research, is clearly of fundamental importance in the relation of cell metabolism and wall deposition. Prior to these later observations the work of Brand (1908) had already suggested the presence of substances with considerable swelling reactions to comparatively mild reagents. A separation of the outer lamella, for instance, could be effected by dilute acetic acid, resulting in a series of 'balloons'. Brand describes this phenomenon as unique among green algae, though it now recalls similar swelling behaviour emphasized in recent times by many workers in certain cells of the higher plant.

With regard to the presence of pectin and the so-called callose, staining reactions show both to be present (ruthenium red or methylene blue for pectin, and picrannil in 4 % sodium carbonate for callose), for the wall stains deeply while controls of pure cellulose (cotton-wool and filter paper) remain unaffected or only slightly stained. Yet treatment appropriate for the removal of these substances has little or no effect on the staining properties of the wall. Thus the walls of all three species stain deeply in picrannil even after 4 days' immersion in 1 % KOH, whereas callose is removed from control sections of *Cucurbita* phloem in a few minutes. A similar treatment in 4 % KOH reduces the depth of stain, under controlled conditions, in the case of *Cladophora prolifera*. Again, the walls stain equally well for pectin before and after treatment with alcoholic HCl followed by 0·5 % ammonium oxalate, or with alternate acid and alkali. Undoubtedly the wall contains some non-cellulosic and non-chitinous substance in considerable amount, but the unreliability of staining reactions render it difficult to determine its precise nature. The evidence would seem to point to the presence of polyuronides (a group which includes the pectins), particularly since they are known to be present in other algae (e.g. polyglucuronic acid in *Fucus serratus*).

The microchemical test for chitin gives rather more positive results, though even here staining reactions often fail. To obtain a good chitin reaction, the material is preferably heated in sealed glass tubes with glycerine to 300° C for a few minutes, and then under similar conditions with 60 % KOH to 180° C for 20 min. The outer layers, particularly of the wall, are then commonly stained a deep violet in the presence of aqueous iodine and dilute sulphuric acid. This is in harmony with the results of Tiffany and Wurdach, and agrees with the results of X-ray analysis.

THE MOLECULAR STRUCTURE OF THE WALL

The principles underlying the investigation of the fine structure of the *Cladophora* wall resemble in their main features those used by the present writers (1937) in the case of the allied genus *Valonia*, except for slight variations in technique on account of the different growth form. Again the chief structural constituent is cellulose, and it is with the organization of this component that we are chiefly concerned here. The terms and concepts used in considering such organization have already been discussed for *Valonia*, but it may perhaps be emphasized that the terms 'micelle' and 'fibril', wherever employed in the present paper, imply regions of more perfect orientation rather than discrete morphological entities.

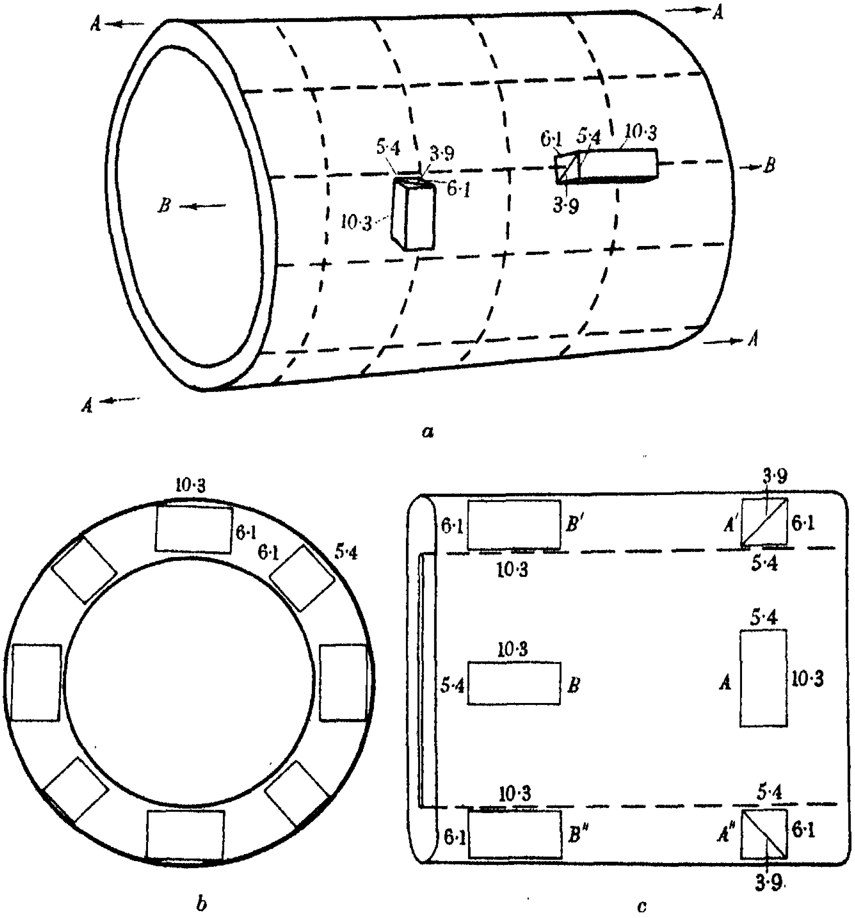
In view of the fact that photographs of compound specimens may be misleading (though there is reason to believe that this is not a serious matter here, see p. 61), it is preferable to present first the diagram given by a single piece of wall, then diagrams given by bundles of filaments.

The internodal cells of *Cladophora prolifera* were found eminently suitable, on account of their relatively large size, for the investigation of single walls. A median portion, some 2 mm. in length and in the form of a short hollow cylinder, was removed from a cell and slit open longitudinally. When mounted in water on a microscope slide, gentle manipulation of a cover-glass sufficed to roll out the cylinder into a flat plate. This piece of wall contained, of course, both inner and outer layers. Its X-ray photograph (figure 2, plate 4) bears a striking resemblance to that given by the *Valonia* wall (Preston and Astbury 1937, figure 9, plate 2). Here again we find two sets of cellulose chains crossed at an angle of some 80° and parallel to two sets of striations visible in the wall (figure 3, plate 4). Moreover, as in *Valonia*, the 6·1 A arc is missing. The planes of 6·1 A spacing must therefore in this case also tend to lie parallel to the wall surface.

Turning now to the photographs given by bundles of filaments, we need consider only *Cladophora prolifera*, since the other species give essentially similar photographs; and it is proposed to confine our attention to the tips of filaments, because here the striation directions vary only slightly from one filament to another and interpretation is therefore less liable to error. In the photographs shown (figures 4–7, plate 4), the majority of the stronger arcs are those of cellulose, and they disappear after treatment of the material with Schweizer's reagent, leaving certain spots typical of chitin. This chitin pattern is to be referred to the wall itself and not to epiphytes upon it, since small pieces of wall free from epiphytes (as observed under the microscope) nevertheless yield good chitin photographs after prolonged exposure.

The conditions under which the photographs were taken may be described first before proceeding to the interpretation. In figure 4, plate 4, the filaments were placed at right angles to the X-ray beam and their length corresponds to the shorter edge of the page. With the beam parallel to the length of the filaments, the diagram obtained is as in figure 5, plate 4. In both these experiments the cells retained their natural cylindrical form. It is, however, in many ways an advantage to use also material which has been flattened, and the photographs from such specimens are presented in figures 6 and 7, plate 4; in figure 6 the beam is perpendicular, and in figure 6 parallel, to the flattened face. In both cases the length of the filaments again corresponds to the shorter edge of the page. Flattening unfortunately produces some angular dispersion among the (previously parallel) filaments, an effect which is quite marked in figure 6, plate 4. As a check, therefore, a photograph was obtained under similar conditions of a single flattened cell.

These diagrams are readily interpreted on the basis of the structure of the wall just indicated. In parts of the filaments near the apices of *Cl. prolifera*, one set of chains forms a rather steep spiral while the other runs very flat, almost in transverse rings. We may therefore assume for the purposes of argument that the cellulose chains are arranged as in figure 8 *a*. Now it is known that four characteristic spacings in cellulose are oriented with respect to the chain direction as in the figure, and with the X-ray beam parallel to the length of the filament these spacings will appear as in figure 8 *b*. For reasons which need not be discussed here the planes of 10·3 A spacing are never recorded as such in the diagram of cellulose and need not be considered. The 6·1, 5·4 and 3·9 reflexions are usually very obvious, however, with increasing intensity in that order. In figure 5, plate 4, therefore, we should expect an intense 3·9 circle in the photograph. On the other hand we may also expect the intensity of the 6·1 reflexion to be greater than that of the 5·4, since the former planes can reflect in both sets of chains while the latter is represented only in the steeper set. This is in complete qualitative agreement with the photograph, in which the intense outer ring represents the 3·9 planes, the inner ring the 6·1 and the less intense ring immediately outside this latter the 5·4.



a

b c

FIGURE 8

Similarly, figure 4, plate 4, is readily interpreted in terms of figure 8 *a*. Along the horizontal line the 6·1 arc is missing, since in both sets of cellulose chains this spacing lies perpendicular to the wall surface. Along the vertical line, however, both 5·4 and 6·1 arcs are plainly visible, the former being reflected from position *BB* (figure 8 *a*) in each cell and the latter from *AA*. Along this line the 6·1 is the more intense arc, since it alone is reflected from both sets of chains. The 3·9 arcs appear as a complete ring for reasons which are clear from a consideration of the case of flattened filaments.

The use of flattened filaments permits us to make a much stricter comparison between photographs of a single wall and those of whole filaments. From figure 8 *a* it is seen that a single flattened cell may be represented diagrammatically by figure 8 *c*. This figure explains at once the photograph obtained: there is no need to reproduce it here, as it is so like that given by a single wall (figure 2, plate 4)—the difference lies in diffraction effects due to the folding at the edge of the cell. The photograph of a bundle of flattened filaments is shown in figure 6, plate 4. In this figure the arcs are drawn out into circles because of an unavoidable angular dispersion in the specimen. The photograph presented in figure 7, plate 4, is also readily intelligible along similar lines. It resembles very closely the photograph of *Valonia* with the beam parallel to one set of chains (Preston and Astbury 1937, figure 10, plate 2), and here again leads to the conclusion that 'the normal to the plane of spacing 6·1 A is confined to about 60° on either side of the normal to the wall surface' (*Valonia*, Preston and Astbury 1937, p. 91).

Thus X-ray analysis of the wall, both of single pieces and of bundles of filaments, demonstrates that the wall of *Cladophora*, like that of *Valonia*, consists of two sets of cellulose chains crossing each other very roughly at right angles. Further, the directions of these chains is indubitably reflected in those of the striations visible under the microscope, both sets of which are in turn visible in both inner and outer layers. In spite of the different growth forms of the two genera, the structures of isolated pieces of wall are almost identical as far as the cellulose component is concerned, though it is difficult to believe that growth form is so independent of wall structure as this might lead us to assume. In point of fact, the similarity of isolated pieces of wall is no guarantee of similarity in the cells as a whole, for the orientation of the isolated piece in the whole wall surface is also of importance; and in general a study of the wall structure of whole filaments of *Cladophora* establishes without doubt an orientation of the chains with respect to the cell axes significantly different from that in *Valonia*.

MICROSCOPIC FEATURES OF THE WALL

The presence of crossed striations in the *Cladophora* wall has been detected by many workers (e.g. Brand 1906). The general opinion seems to be that the lamellae alternate in striation direction, and with this we are in complete agreement. The appearance on the wall of striations coinciding in direction with the cellulose chains is particularly fortunate. It is not possible at the moment in dealing with filamentous algae to survey the structure of a whole filament by an X-ray method, but observation under the microscope can be used to advantage, particularly since the size of the filaments permits a comprehensive series of determinations without the uncertainty resulting from dissection. The filaments used were observed in water, and the drawings reproduced below were made with the aid of a camera obscura. For clearness of detail these drawings were later conventionalized, with no change of scale, the angles of branching being made more regular and much larger. The striations apparently represent real structural discontinuities in the wall substance. When tension is exerted on a filament so that it breaks in two, the wall lamellae with the steeper striations appear to break first. As the two pieces of filament are then moved apart, fibrils 'unwind' from the layers built from the flatter spiral, and both ends rotate rapidly. The separation of the two pieces may go on over considerable distances, leaving a continuous strand of fibrils (figure 9, plate 4).

The visibility of the striations varies considerably even in different cells of the same filament. Although often due to the presence of opaque granules in the fixed and cleared cytoplasm, this variability is frequently to be explained only by some change in the wall itself; yet failure to observe striations of any kind is always to be ascribed to the presence of opaque material. On the other hand, striations are always lacking on cross-walls, and are detected with difficulty at the extreme tips of apical cells. Undoubtedly, the pronounced hemispherical curvature of the tip of an apical cell, involving as it does extreme accuracy in focussing, explains in part the failure to detect both sets of striations in this region, where up to the present only the longitudinal set has been clearly observed. The rest of the filament shows both striations running as uninterrupted spirals round the cells and the filaments as a whole, one set following a moderately flat and the other a steep spiral of opposite sign. Occasionally the two spirals, still of opposite sign, have almost the same inclination. Under the polarizing microscope, the younger cells (three or four behind the tip) are isotropic, or only feebly anisotropic, and uniformly so; whereas older cells almost invariably show small mosaic areas, similar to those noted for *Valonia* (Preston 1931), except that they frequently tend to take the form of transverse bands—a fact which may be of considerable interest. It is further significant that the steeper set of striations is always the more readily visible, although the uniformly feeble anisotropy of younger cells would point to almost equal proportions of the two sets of chains.

A survey of the wall structure of the whole filament brings out several facts of importance for a consideration of the growth processes of the plant.

In only one species, *Cl. prolifera*, does the striation direction of a cell show any obvious correlation with its position in the filament. Here, although the inclination of both spirals varies from point to point in a cell, there is a general tendency for the steep spiral to be steeper the nearer the cell is to the tip of the filament. This will be clear from figure 10 and table 1. No

TABLE 1. THE INCLINATION IN DEGREES TO THE LONGITUDINAL AXIS OF STRIATIONS IN FILAMENTS OF *CLADOPHORA PROLIFERA*

A negative sign indicates a left-hand spiral, the absence of sign a right-hand spiral.
a = end of cell nearer tip of filament.
b = centre of cell.
c = end of cell farther from tip.
Spaces left blank indicate that the striations are obscured.

	Inclination						Inter-striation angle		
Cell	*a*		*b*		*c*		*a*	*b*	*c*
1	0·0	89·5	−20·0	76·0	−33·5	60·5	89·5	96·5	93·5
1·1	—	—	0·0	74·0	0·0	—	—	74·0	—
1·2	− 9·5	76·5	−18·0	76·5	−23·0	76·5	86·0	94·5	99·5
1·21	—	—	—	—	− 4·0	85·0	—	—	89·0
1·22	—	—	—	—	−20·0	70·0	—	—	90·0
1·23	—	—	—	—	−11·5	75·5	—	—	87·0
1·3	− 5·0	79·0	− 4·0	86·0	−10·0	68·5	84·0	90·0	78·5
1·31	—	—	− 3·5	—	− 3·5	78·5	—	—	82·0
1·311	0·0	—	0·0	—	− 6·0	—	—	—	—
1·32	0·0	—	− 2·0	72·5	− 3·5	79·5	—	74·5	83·0
2	−10·5	—	−28·0	67·0	−15·0	65·0	—	95·0	80·0
2·1	0·0	83·5	−13·0	78·0	− 7·0	77·0	83·5	91·0	84·0
2·11	—	—	—	—	− 6·0	72·5	—	—	78·5
2·2	0·0	75·0	—	—	−21·5	67·0	75·0	—	88·5
2·21	−10·5	84·5	−12·5	—	−19·5	72·0	95·0	—	91·5
3	0·0	—	− 9·0	84·0	−18·0	73·0	—	93·0	91·0
3·1	− 3·0	81·0	−10·0	81·0	−11·0	80·0	84·0	91·0	91·0
3·11	− 8·5	—	− 4·0	74·5	− 3·0	—	—	78·5	—
3·111	0·0	—	0·0	—	8·0	98·0	—	—	90·0
3·112	− 9·0	72·5	− 9·0	72·5	− 9·0	72·5	81·5	81·5	81·5
3·12	− 2·5	—	− 7·5	84·0	− 8·5	85·0	—	91·5	93·5
4	− 6·5	—	−14·5	74·0	− 4·5	—	—	88·5	—
4·1	− 8·0	—	− 4·0	—	− 0·0	—	—	—	—
4·11	0·0	83·0	− 3·5	—	−10·5	73·0	83·0	—	83·5
5	− 7·0	—	−11·0	72·5	−10·0	79·0	—	83·5	89·0
5·1	0·0	87·0	0·0	85·5	− 8·0	76·0	87·0	85·5	84·0
6	0·0	90·0	−17·5	73·0	—	—	90·0	90·5	—
6·1	0·0	90·0	0·0	91·0	−11·0	—	90·0	91·0	—
6·2	0·0	78·0	− 5·0	72·0	−20·0	71·0	78·0	77·0	91·0

The cells are numbered in accordance with figure 10.

such generalization can be made with either of the other species, though the variations in inclination within a single cell are perhaps sufficiently uniform to have some significance. The rule seems to be that whenever considerable changes in inclination of the striations occur, the steeper spiral is steepest at the end of a cell nearer the tip of a filament, and least steep towards the middle or, more often, the other end of that cell. The condition of the flatter spiral is always the reverse—flatter nearer the tip.

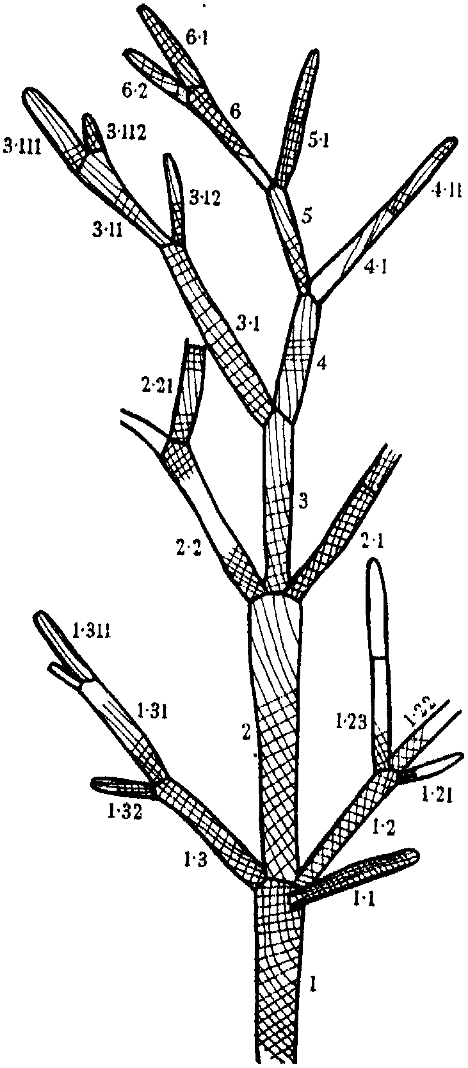


FIGURE 10. (Magnification × 12·5.)

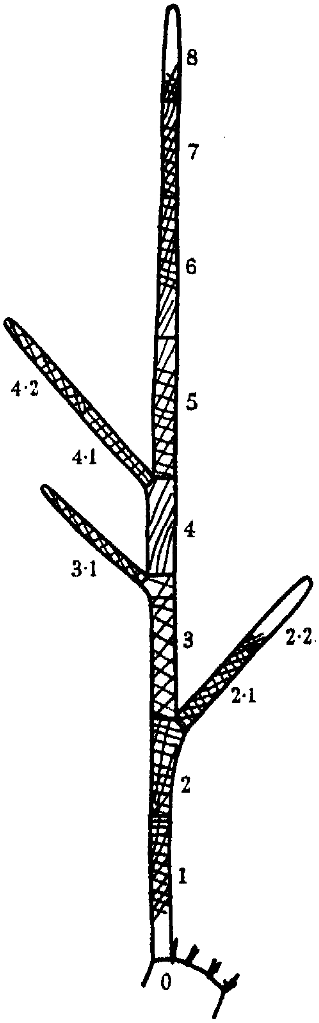


FIGURE 11. (Magnification × 12·5.)

Here again these conditions are more marked in *Cl. prolifera*, in which few exceptions occur. It is, in fact, a striking property of this species that the striations of the steep spiral generally become approximately parallel to the long axis of the cell immediately below a cross wall. In this species also, the tip cells are peculiar in that one set of striations is usually parallel to the whole length of the cell, and this is a very common feature. The possibility of a connection between these extreme manifestations in *Cl. prolifera* and the frequent branching is sufficiently obvious to need little emphasis.

Variations of the same kind may be observed in both collections of *Cl. arcta* (although their growth forms are slightly different), where, however, more frequent exceptions occur (figure 11 and table 2). But a phenomenon becomes apparent in this species which is masked by the change in striation direction observed in *Cl. prolifera*. A considerable change is usually observed on the outer layer of the wall, enclosing the whole filament, at points where cross walls have been laid down. At these positions, the striations of the steeper spiral become oriented approximately parallel to the length of the filament. Only this one set of striations can commonly be observed here, the presence of the second set being masked by the coarser lamellation of the underlying cross wall. Both sets, however, are evident in *Cladophora gracilis* (which for the reason given below does not show the phenomenon mentioned above), in some of the preserved material of which the (double) cross walls have pulled apart, leaving the outer layer free for observation (figure 12, plate 4). This outer layer clearly possesses both sets of striations, although the preponderance of the steep set over the less steep would appear to be greater here than in the whole wall thickness.

The behaviour of the flatter spiral is, in general, the reverse of the steeper. When the latter becomes steeper the former commonly becomes flatter. This promises to be a point of extreme importance in future discussions of wall deposition and growth, and it seems worth while to enlarge upon it a little. The observations refer primarily to *Cladophora prolifera* and *Cl. arcta*, since in *Cl. gracilis* we have a situation similar to that experienced with *Valonia*—one of the sets of striations is nearly always parallel, and the other perpendicular, to the cell axis. In the two first-mentioned species, it is possible to establish a correlation between the inclination of the two spirals, measured from the longitudinal axis of the cell. Taking both inclinations as positive, the correlation coefficients, calculated from tables 1 and 2 are as follows:

Cladophora prolifera	−0·59
Cladophora arcta (Filey)	−0·55
Cladophora arcta (R.H.B.)	−0·68

TABLE 2. *CLADOPHORA ARCTA*. DETAILS AS IN TABLE 1

(a) Robin Hood's Bay Collection*

Cell	Inclination						Inter-striation angle		
	c		b		a		c	b	a
1	—	—	−28·0	83·0	− 7·0	87·0	—	111·0	94·0
2	−20·0	78·0	−16·0	80·0	−20·0	79·0	98·0	96·0	99·0
2·1	−22·0	73·0	−12·5	73·5	− 9·0	83·0	95·0	86·0	92·0
2·2	−15·0	81·0	—	—	—	—	96·0	—	—
3	−20·0	—	−30·0	66·0	−32·0	76·0	—	96·0	108·0
3·1	− 9·5	85·0	—	—	−10·5	82·5	95·0	—	93·0
4	−22·5	—	−30·0	—	−24·0	—	—	—	—
4·1	−14·0	80·0	−15·5	—	−12·0	82·0	94·0	—	94·0
4·2	−22·0	79·0	−13·5	82·0	−10·0	78·0	101·0	95·5	88·0
5	−27·8	80·0	−27·5	80·0	−21·0	—	107·8	107·5	—
6	−15·0	—	− 8·0	81·0	−13·0	78·0	—	89·0	91·0
7	−20·0	74·0	−19·0	73·5	−13·0	—	94·0	92·5	—
8	− 9·5	80·0	—	—	—	—	89·5	—	—

(b) Filey Collection†

Cell	a		b		c		a	b	c
1	8·5	−72·0	20·0	−80·0	20·0	−83·0	90·5	100·0	103·0
1·1	17·0	−80·0	12·5	−80·0	14·0	−80·0	97·0	92·5	94·0
1·11	12·5	−80·0	—	—	14·5	−80·0	92·5	—	94·5
1·12	18·0	—	16·5	—	20·0	—	—	—	—
1·13	12·0	−82·0	16·0	−82·0	23·5	−82·0	94·0	98·0	105·5
1·14	9·0	−80·0	20·0	−78·5	12·0	−83·0	89·0	98·5	95·0
1·15	8·0	−81·0	—	—	17·0	−82·5	89·0	—	99·5
1·16	15·0	−80·5	—	—	5·0	−85·0	95·5	—	90·0
1·17	10·0	−80·0	—	—	17·0	−80·0	90·0	—	97·0
1·18	10·0	−82·0	16·0	−81·0	17·0	−76·0	92·0	97·0	93·0
1·19	19·0	−80·0	15·0	−79·0	17·0	−79·5	99·0	94·0	96·5
1·2	9·0	−84·0	13·0	−87·0	5·5	−88·0	93·0	100·0	93·5
1·21	24·0	−83·5	—	—	23·0	−83·5	107·5	—	106·5
1·22	13·0	−86·0	—	—	13·0	−81·5	99·0	—	94·5
1·23	18·0	—	—	—	23·0	−86·0	—	—	109·0
1·24	6·5	−80·0	20·0	−82·0	26·0	—	86·5	102·0	—
1·25	9·0	−85·0	—	—	16·0	−85·0	94·0	—	101·0
1·26	—	—	18·5	−78·0	20·0	−78·0	—	96·5	98·0

* See figure 11.

† This table represents a cell (1) with two branches, one of ten cells (1·1–1·19) and one of seven cells (1·2–1·26).

Interpretations, in absolute terms, of isolated correlation coefficients is, of course, always somewhat uncertain. The figures are considerably greater than zero, however, and we may tentatively suggest that the two sets of striations do show the kind of variation outlined above. In other words, the two sets so adjust themselves that the angle between them tends to remain constant, though environmental conditions limit the effectiveness of the adjusting mechanism. It may be further significant that for all specimens observed the average angle of representative samples is about 83°—the same figure as that recorded for *Valonia*—and that this angle is definitely less than a right angle. This fact cannot be over-emphasized in view of the increasing prevalence of certain hypotheses regarding problems of wall deposition, to be discussed later in this paper. Whether the precise value of the interstriation angle has any significance is not clear, especially since the figures from certain cells of the higher plant are considerably less than 83° (Preston 1939 *a*).

That the portion of the wall immediately below a cross wall is in some way different from the rest is suggested by purely morphological considerations. It is from this part only of the wall that branches arise, and this region alone is responsible for the emission of zoospores (Brand 1901). By analogy with other filamentous algae one may, in fact, conclude that this is the only region of the wall which continues to elongate appreciably after the cell is cut off from the growing tip. This fact is well recognized in the literature (e.g. Brand 1901) and may readily be demonstrated by the method used by Noll (1887) for *Caulerpa* and other siphoneous green algae. In this method, living material is immersed for a short time in ferric chloride solution and then transferred to potassium ferrocyanide. A deposit of Prussian blue is thus produced *in* the wall which, if the staining has been carefully performed, does not impair cell activities. With *Cladophora*, solutions consisting of one part of 3 % $FeCl_3$ solution to one part of sea water, and an equal strength of $K_4Fe(CN)_6$, were found to produce a deep coloration in the wall. On replacing the plant in the culture tank, after a brief washing in sea water, the colour rapidly disappeared, and even after 8 weeks the plants differed in no way from the untreated controls. One week after staining, part of the treated plants was removed and the colour developed with acidulated ferrocyanide. Only at the tips did the wall appear uncoloured, so that only in this region had any new wall substance been developed (dead plants remain coloured to the very tips). In successive weeks, as the tip cell elongated, the uncoloured zone spread until about one-half of the tip cell appeared uncoloured at the end of 4 weeks. Simultaneously, a slow progressive weakening of the colour of the upper part of the walls of the second and third cell from the tip (*a* and *b* in figure 1) was observed, together with an occasional weakening at localized regions in older cells. Only in such regions as show this gradual disappearance of colour may we assume with certainty that the wall is increasing in area.

The nature of the wall at the growing tip is clearly of paramount importance in a discussion of the growth of *Cladophora*. The evidence has been seen to point to a comparative lack of transverse striations at the growing tip, though this flatter set is everywhere the less easily visible and therefore, as already pointed out, failure to observe striations in these regions is no proof of their absence. Evidence of an indirect nature does, in fact, point to the presence of both sets at the apex. Both sets of striations are readily visible in the lower portions of the apical cell, and the wall layers in this region are observed to pass completely round the tip itself. Further, under the polarizing microscope the alternation of light and dark lamellae caused by the alteration in chain direction is as obvious at the tip as in the rest of the wall. In view of the unlikelihood of any abrupt change in chain direction in a single layer (for those regions showing both striations were originally part of a tip), the conclusion seems inevitable that the tip is indeed clothed with layers having both sets of cellulose chains. This view is further supported by the fact that the whole of the wall of a tip cell is isotropic in face view, whereas we should expect pronounced anisotropy if one set of chains were absent.

BRANCHING

The structure of the wall of *Cladophora* thus outlined refers, as has already been pointed out, only to cells free from branches or, if branched, to those parts of the wall sufficiently removed from the branching region. It is to be expected that the presence of a branch will cause some modification of wall structure, and such is indeed the case. The phenomena involved in the development of a branch have received some attention at the hands of Rosenvinge (1892), Nordhausen (1900), and Brand (1901), but the effect on the fine structure of the wall has been largely overlooked.

It is characteristic of *Cladophora* that branches are emitted only from the upper end of a cell. They are first evidenced as slight lateral swellings in the second or third cell from the tip, which rapidly develop, apparently through the agency of a secondary growing apex, into an elongated structure closely resembling the apical cell of the parent filament. The proximity of a branch causes some modification of wall structure in the parent cell.

The three species used here are essentially similar in this respect, and it will be sufficient to discuss only the simplest (*Cl. gracilis*) in any detail. Observation of young branch initials and of the point of union with the mother cell is peculiarly difficult, since at these regions considerable masses of opaque granules occur in fixed material. The transverse striations are extremely difficult, and often impossible, to detect, and even the more obvious longitudinal set frequently becomes obscured. The following observations were made on the comparatively few branch regions which were sufficiently free from these defects, and on those from which the granules could be removed without serious damage to the wall.

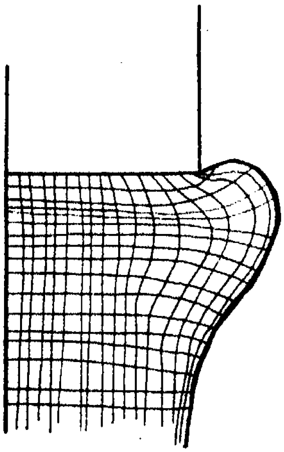


FIGURE 13

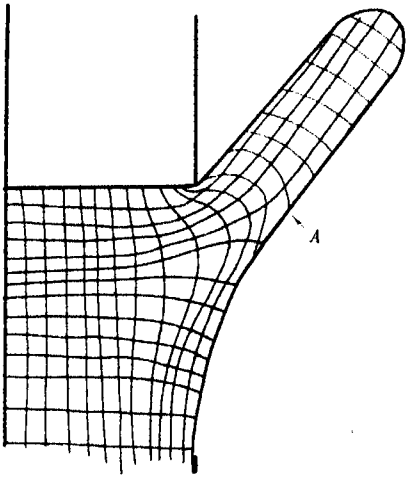


FIGURE 14

A young branch initial appears as in figure 13. The longitudinal striations bend towards the initial exactly as would parallel lines ruled on an originally cylindrical balloon manufactured so as to inflate laterally near one end, and the transverse striations clearly pass over in the manner indicated. At a somewhat later stage the striations of an outer lamella may still be seen surrounding the branch initial taking the form indicated in figure 14. The longitudinal striations on the parent cell become the transverse set of the branch, and the transverse set becomes the longitudinal. This condition clearly results from the continuation of the mechanical factors which played a part in the origin of the branch, and is maintained in this outer layer without serious modification (see below).

In older branches, however, the newly deposited inner lamellae show an essentially different configuration. Here the longitudinal striations pass over gradually from the parent cell to the branch; and although the transverse striations are difficult to observe at the base of the branch itself, the run of these striations at the point of branching suggests that they are derived from the corresponding set in the parent cell (figure 15). On the other hand, some of the longitudinal striations on the upper part of the branch certainly link up with the transverse striations of the parent. At this time, careful observation reveals that an outer lamella as in figure 14 is still intact. This state of affairs persists until the branch cell has become so elongated that in the outer lamellae even only a short way from the node, no connection can be traced between the longitudinal striations of the branch cell and the transverse set of the parent. The latter can be traced to A (figure 14) and cease there: the striations further along the branch, at the same focus, are still longitudinal but are connected with the longitudinal striations of the parent cell. The rapid elongation of the branch initial has thus involved an attenuation or even a complete fracture of the outer lamellae constituting the outer layer of the wall. That such fractures do occur is suggested by observation on living material placed in distilled water. The filament develops a turgor pressure sufficient to burst open some of the cells. Commonly the second, third, or fourth cell from the tip expands most rapidly and pushes the cells before it out of the filament. Although the external surface of the cells thus extruded is quite smooth, a delicate cylindrical protuberance is always observed connected to the remainder of

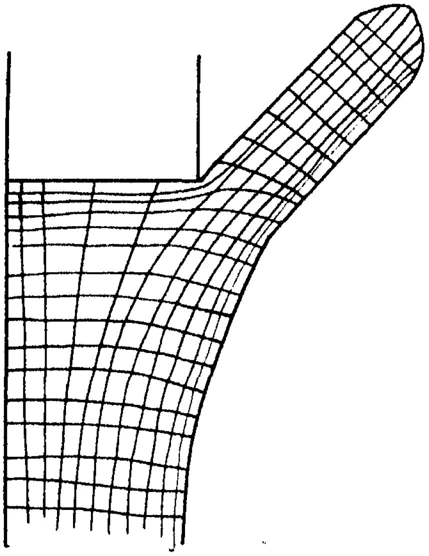


FIGURE 15

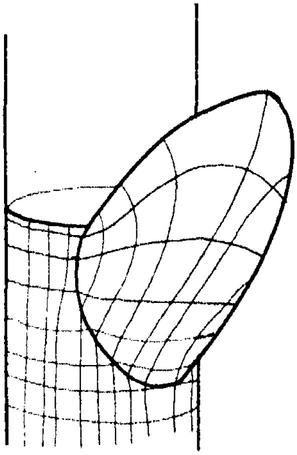


FIGURE 16

the filament and continuous with the outer lamella clothing the tip. It gives some indication of the usual cellulose test (though the colour is violet rather than blue), but shows no striations and is optically isotropic in face view. Failure to observe striations is doubtless due to its extreme tenuity.

That growth processes are also involved is evidenced by the fact that the original transverse striations of the branch cell (originating from the longitudinal set of the parent) show no tendency to become longitudinal as they would inevitably do by purely mechanical extension. The tip of a branch initial does, in fact, early assume the characteristics of a typical apical growing point.

The sequence of events in the other species is essentially the same except that we have here, especially in the case of *Cladophora prolifera*, the complication that the lower half of the branch cell often becomes spiral (figure 10).

Discussion

Thus once again we meet, in these lower forms, the spiral organization which is so common a feature in plants. The conclusion is unavoidable that this prevalence of spiral structure reflects some underlying vital principle that is common to the whole of the plant kingdom. At the same time, spiral structure is encountered so frequently, and in so many and diverse types of material, as to imply a multiplicity of causal mechanisms. The geometrical principles underlying such structures are undoubtedly similar everywhere, though the precise manner in which they come to be applied probably varies; but wherever, as in the present case, modifications occur, we may be sure that they are intimately associated with corresponding changes in the physical and chemical factors conditioning growth—that wall structure is, in fact, influenced by growth form. That the converse is true during some phase of development is also to be remembered. For instance, an elongated cell with a single spiral winding will continue, under suitable conditions, to increase in length rather than in diameter—wall structure conditions growth. Simultaneously, this elongation may cause a steepening of the spiral—growth conditioning wall structure.

Continuing this line of argument, it should be possible eventually to trace a specific connexion between the growth processes of a cell and its particular wall structure at any moment. An attempt to establish such a connexion will preferably commence with an examination of young material, and this is perhaps particularly true in the filamentous algae. At present, however, such material is not available in these laboratories, so that it seems unprofitable to indulge in any detailed speculation just now. Yet even in this work on what may be termed the "adult" material we may glimpse, very hazily it is true, the general outline of the processes at work on the wall during cell development.

TABLE 3. TURGOR PRESSURES, IN ATMOSPHERES, OF CELLS IN *CLADOPHORA ARCTA**

Position of cell numbered from the tip	Turgor pressure
1	16
2	19
3	17
4	18

* Turgor pressures were determined in the laboratory at Robin Hood's Bay by

(*a*) obtaining the point of 50 % plasmolysis in sugar solutions made up in sea water, and

(*b*) assuming the suction pressure of the cells to be equivalent to the osmotic pressure of sea water.

In the calculations, allowance was made for the dilution of the sea water in the sugar solutions. Results are given to the nearest 0·5 atmosphere. One hundred cells of each kind were examined in each solution.

These results were obtained during midsummer. Rough redeterminations in the following spring revealed a much wider variation of the same kind.

The facts may be summarized very briefly. Passing from the base of a plant to the tip of the filament, *where the walls have, in effect, undergone more elongation*, we observe quite generally a gradual straightening out of one spiral in the wall. In individual cells, this same spiral is steeper nearer the tip end of the cell, *again in a region which is more elongated.* Certainly there are exceptions, particularly to the latter rule; but it is not impossible that anomalous local steepening goes hand in hand with anomalous elongation, and in any case these exceptions should not be allowed to obscure the main principle. *Elongation* could therefore be postulated as the direct causative factor for this aspect of the observed effects. This would bring *Cladophora* into line with cells in the higher plant, where similar processes are at work (Preston 1934, 1938, 1939 *a*, *b*). Now the hydrostatic pressure inside the cells of *Cladophora* is considerable, and it is surely significant that almost invariably this pressure is greater in the second cell than elsewhere in a filament; for it is in this particular cell that the bulk of the extension occurs after the first division. It might therefore be reasonably inferred that internal pressure, leading to elongation of the spirally wound wall, is responsible for the progressive steepening of one spiral. The same process is also undoubtedly at work in steepening this spiral in the region where a transverse wall occurs (p. 67).

The converse behaviour of the second spiral would also appear to receive explanation along these lines, for we have seen that the mechanism of wall deposition, presumably protoplasmic as already suggested for *Valonia* (Preston and Astbury 1937), involves a periodic change, through a definite angle, in the direction of cellulose chains. Thus, wherever one spiral becomes steeper, the other must become flatter. There must of course, during further elongation, be some slight readjustment in the expanding layers already present, and this may account for the observed variation in the interstriation angle; but once a cell has ceased to elongate, the continuity of the protoplasmic mechanism will maintain this angle at its constant value in subsequent layers. The process of elongation may also bring about this converse behaviour of the second spiral in a different way. Continuous elongation of the filament tip, especially under the influence of internal pressure, seems to involve rotation of the tip, and, in fact, rotation has been observed. It would tend inevitably to steepen one spiral and flatten the other.

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DESCRIPTION OF PLATE 4

FIGURE 2. X-ray photograph of a single piece of a wall of *Cladophora prolifera*. The X-ray beam is normal to the surface.

FIGURE 3. Photomicrograph of the piece of wall used in obtaining figure 2. Notice the two sets of striations which are perpendicular to the two rows of diffraction spots in figure 2.

FIGURE 4. X-ray photograph of a bundle of filaments with the X-ray beam perpendicular to their lengths.

FIGURE 5. X-ray photograph of a bundle of filaments with the X-ray beam parallel to their length.

FIGURE 6. X-ray photograph of a flattened bundle of filaments with the X-ray beam perpendicular to the flattened face.

FIGURE 7. As in figure 6, except that the beam is parallel to the flattened face and perpendicular to the length of the filaments.

In figures 4, 6 and 7 the bundle of filaments lies parallel to the shorter edge of the page.

FIGURE 9. A filament of *Cladophora arcta* broken by longitudinal tension. A fine strand can be seen still uniting the two halves of the filament. This has been "unwound" from the layers with the less steep striations.

FIGURE 12. A cross-wall in a preserved specimen of *Cladophora gracilis*. The two layers of the cross-wall have become separated, and both sets of striations can be seen in the outer layer uniting the two cells.

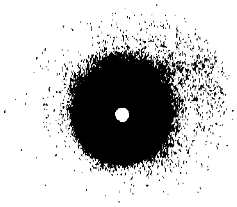

FIGURE 4

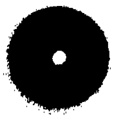

FIGURE 5

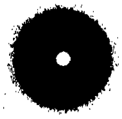

FIGURE 6

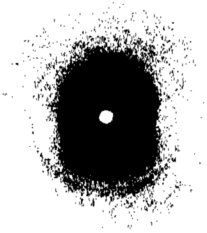

FIGURE 7

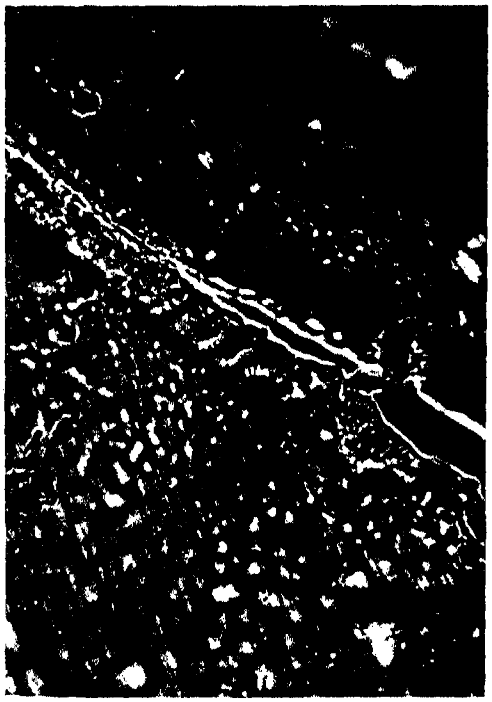

FIGURE 3

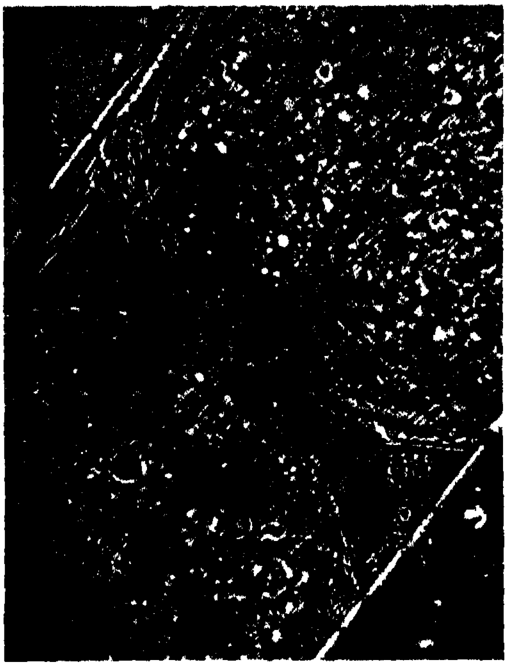

FIGURE 12

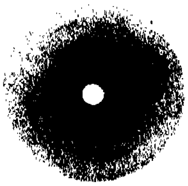

FIGURE 2

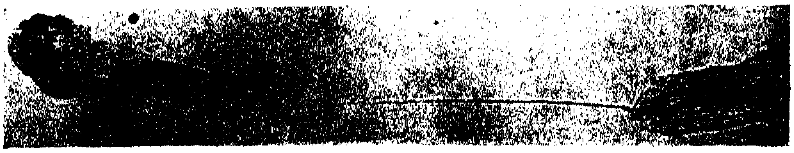

FIGURE 9

On a green helical organism and its motion

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[Plates 5, 6]

A green helical organism is described which displays a new type of movement. The helix moves by growing at the anterior end and diminishing posteriorly; it does not rotate. Observations on the change in shape produced by dehydration show that the structure of the organism is markedly anisotropic. By analogy with other fibrous structures, it may be concluded that anisodiametric micells or molecules are orientated parallel to the long axis. It is pointed out how the growth and activity of the organism are related to this axis.

The systematic position of the organism is discussed and its possible affinity with the Spirochaetoidea (Dobell) and Cyanophyceae considered.

1. Introduction

The organism to be described here appeared in a sample of material from a small aquarium covered with duckweed. It was first noticed in December 1938 and was fairly plentiful until the beginning of January 1939, when it disappeared, in spite of the addition of a nutrient medium (that of Benecke; see Doflein-Reichenow 1928). The organism has not since then been found in the aquarium from which the sample was taken. An examination of the literature for previous records of such a green spiral-shaped protist has been made, but no description has as yet been found which would suggest that the organism has been observed before. The movement exhibited is so remarkable and can be observed with such ease that it is scarcely conceivable it should have escaped notice if the organism were indeed identical with any of the green spiral organisms hitherto described. The present paper offers a preliminary account. Until the reappearance of the organism no attempt will be made to settle its systematic position; as will be seen, it appears to be closely related to the Spirochaetoidea (Dobell 1912) and to certain Cyanophyceae. This account will be concerned for the most part with a description of the new type of movement which it exhibits and with some reflexions on organization and activity.

2. THE GREEN HELICAL ORGANISM

The organism is a transparent, light green ribbon, wound in a close, right-handed helix. The diameter of the tube thus formed is between 1·6 and 2·0 μ; its length varies between 10 and 18 μ. The edges of the ribbon bear a regular series of highly refractive granules, spaced at intervals of about 1 μ; these were particularly conspicuous in certain individuals (as in plate 6), but much less so in others (figure 3, plate 5).

Dobell (1912) rightly questions accounts given by Lagerheim (1892) of green spirochaetes about 2 μ wide, pointing out that it is necessary to know the nature of the optical equipment with which the observation was made before passing judgement on the reliability of the observation. The green helical organism described here was examined with a Leitz 2 mm. apochromatic objective (n.a. 1·32, homogeneous oil immersion) in conjunction with Leitz periplanatic eyepieces (× 6, × 10 and × 20), and with an achromatic condenser of numerical aperture 1·40. The green colour is a bright green, not the typical blue-green of the Cyanophyceae, and disappears during alcoholic dehydration of the preparations. It was observed that the last surviving specimens were distinctly paler in tint than those first observed. Since the colour was not at all the faint apple green seen in colourless objects as a result of the dispersion of even the finest objectives, it seems certain that the colour is due to some alcohol-soluble pigment. The organism was observed and the colour confirmed by many members of this department.

Nothing is known concerning its mode of nutrition. It was often observed that bacteria became attached to the surface (see p. 81) but these were never seen to be ingested. The free-living spirochaete, *Spirochaeta fulgurans* Dobell (?), was present in large numbers in the culture, but conditions were not markedly saprobic, since numerous minute green algae were found, together with colourless flagellates belonging to the genera *Bicoeca* and *Salpingoeca*. The significance, if any, of the green pigment is unknown.

Division was never observed, but it appears extremely probable that transverse fission occurs. The organisms are always of approximately the same width but differ in length, the longest being about twice as long as the shortest. On one occasion two individuals were found placed as in figure 1 *b*. They separated immediately and moved away from each other. This position is very similar to that figured by Gross (1910), figure 28, Tafel 3, in the division of *Cristispira*. It is suggested that this pair may have resulted from the constriction of a single, double-length individual.

Permanent preparations were made using Schaudinn (with acetic acid) and Champy as fixatives and staining with Mayer's haemalum and eosin, iron haematoxylin and orange G, and Feulgen and light green. Preparations were obtained by covering a drop of the culture with a no. 0 cover-slip and observing under oil immersion until the helical organism and spirochaetes, *Spirochaeta fulgurans* Dobell (?), were crawling on the surface of the cover-slip or slide. A drop of fixative was then placed at the edge of the cover-slip and the volume of liquid was increased gradually until the cover-slip floated off and could be transferred to a watch-glass for further treatment. The slide was also stained. In this way preparations could be obtained without the use of egg albumin. The helical organism was never very abundant in the sample, but, using this method, very few of those attached to the glass at the moment of fixation were lost during subsequent treatment. It was also possible by this means to follow the changes in shape of the organism during fixation and dehydration; these observations will be referred to later (p. 87).

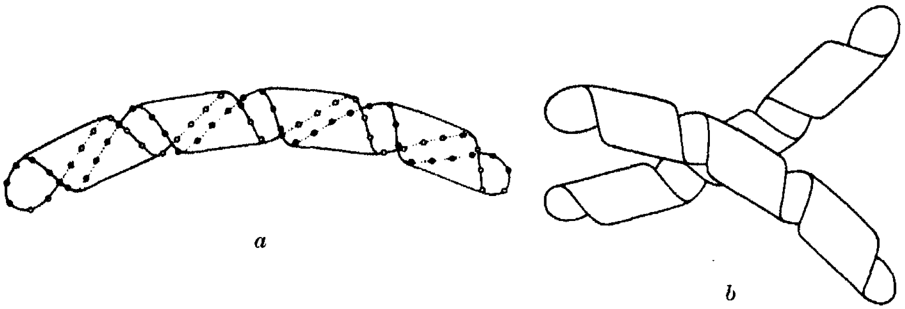

FIGURE 1. (*a*) A somewhat diagrammatic representation of the green helical organism, showing the disposition of the refractive granules along the edge of the helically twisted ribbon. For the sake of clarity the granules are represented rather smaller, relatively, than in the living organism. Granules which are stationary relative to the background are represented by black circles, those in motion by white circles. (*b*) Two short individuals which may have arisen by transverse fission (see p. 78). This figure should be compared with that showing fission in *Cristispira* in Gross (1910). The two individuals discovered in this position subsequently separated.

None of the methods of fixation and staining used revealed any trace of chromatic material. Material fixed with Schaudinn or Champy and stained with Feulgen gave negative results, although colourless flagellates present were satisfactorily stained. Preparations treated with Feulgen's reduced *Diamantfuchsin* did not show the granules visible in life, nor were these visible in any of the permanent preparations. They could not be shown to be volutin, as are the granules in *Spirochaeta plicatilis* Ehrenberg (Zuelzer

1925). Iron haematoxylin preparations were uniformly grey in tone. Figure 4, plate 5 shows permanent preparations fixed with Schaudinn and stained with iron haematoxylin and orange G.

3. MOVEMENT

The organism exhibits two types of movement: (1) helical crawling, and (2) slight flexures of the body which cause the path to deviate from a straight line (see plates 5 and 6). The rate of movement is much slower—both absolutely and relatively—than that of typical spirochaetes. This can be determined most accurately from the cinematographic films. The films were taken at a speed of 8 pictures a second under oil immersion with a × 20 eyepiece. The camera and microscope were supported independently. The light-source was an arc, and the beam was passed through a copper sulphate solution and through a green Wratten filter, no. 58, which was selected empirically, on the grounds that only in its presence did the organism remain active for a period of several seconds. Without this filter, movement stopped immediately. The exposures were made with a squared micrometer disk in the eyepiece. At the magnification used, the side of each square in plates 5 and 6 is $7\cdot5\mu$.

From the films it is found that the organism travels its own length in about 20 sec., that is, comparatively slowly. Hence it is possible to observe with ease what is happening. The key to the type of movement which has been described as helical crawling is given by the highly refractive granules. Those on the edge of the ribbon directed towards the direction of movement (see figure 1*a*) are observed to be streaming forward relative to the background and to the anterior or posterior ends of the organism. If a single granule is followed forward, it is observed to become stationary relative to the background as soon as it reaches the anterior end; relative to the ends of the organism, however, it now travels backwards to the posterior end and then moves forward relative to the background once more.

The movement is most easily pictured as that of a helically-wound caterpillar tractor, one edge of which is motionless relative to the substratum (see figure 2*b*). The treads (that is, the granules) on the moving edge advance twice as rapidly as the anterior end. The green helical organism does not rotate about the axis along which it is moving, but progresses by adding to the helix at the front end and reducing it behind. If attention is confined to those coils of the helix which lie between the two ends, these are found to be motionless with respect to the background. The anterior tip however describes a helix as it grows, while the posterior tip traces one as it withdraws.

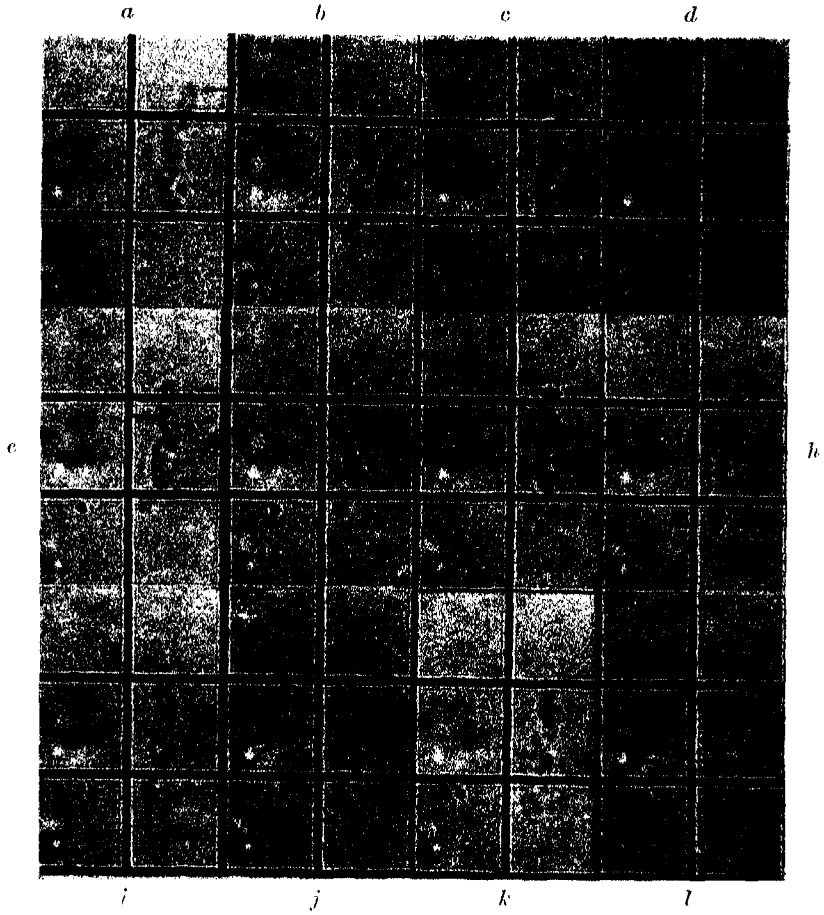

FIGURE 3. Shots taken at the rate of 8 pictures a second. Those selected are in order from left to right and from above downwards nos. 2, 5, 9, 16, 20, 22, 24, 26, 31, 34 and 38. The set of photographs represents therefore a time interval of about 5 sec. It will be noticed (see figure *i*, for example) that the helix appears to be left-handed. This is because the lower parts of the coils are in focus; at a slightly higher focus (see plate 6) the helix would be seen to be right-handed. The granule marked → passes from the posterior end forward, about half-way along the body, following the anterior edge of the helix. In figures *e*–*j* a stationary granule, *X*, is visible on the posterior edge of the helix. Magnification × 1700.

FIGURE 4. Three specimens fixed with Schaudinn and stained with iron haematoxylin. Notice the change in proportions resulting from marked anisotropic shrinkage on dehydration. The helical shape persists but the pitch of the helix increases. Magnification × 1500.

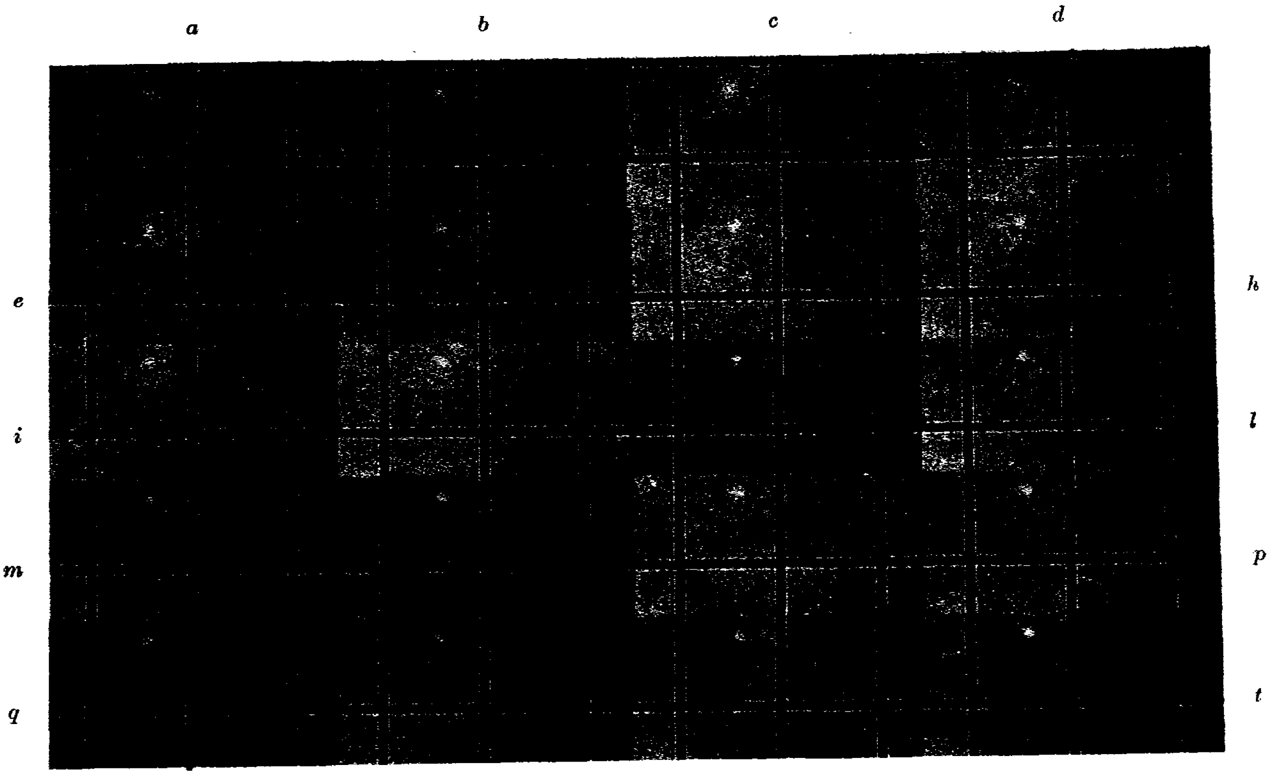

FIGURE 5. Shots taken from a sequence obtained from a large individual with very distinct granules. From left to right and from above downwards the serial nos. are 7, 32, 37, 41, 48, 51, 53, 54, 56, 64, 68, 70, 74, 77, 79, 82, 84, 86, 87, 90. The organism has advanced therefore through about half its length in 11 sec. This sequence shows the change in appearance of the granule (marked by arrow) due to its vertical movements. In figure *a* it is still stationary; in figure *b* the organism has moved on until the stationary granule is terminal; in figure *c* it begins to move forward and changes its vertical position (lower focus), now appearing black, figure *d*; in figure *e* it reappears and moves along the anterior edge of the helical ribbon until it passes out of sight in figures *h* and *i* (notice that the helix is right-handed in these photographs); in figure *j* the granule reappears and goes through the same cycle, becoming black (that is, passing beneath the upper turn) in figure *m*; it reappears in figure *o* and becomes black again in figure *s*. Magnification × 1700.

The movements of single granules can be followed in figure 2. The most anterior stationary granule in figure 2*a*, I is marked 0; proceeding from this granule the six most anterior moving granules are numbered in sequence: 1, 2, 3, 4, 5 and 6. The drawings (I, II, III, IV, V and VI) are made at equal intervals of time, such that between each drawing each of the moving granules moves along the edge of the helix through a distance equal to the distance between any two adjacent granules (the granules always remain the same distance apart). In *a*, II granule 1 at the anterior end has moved into the position which it will continue to occupy until it is once more at the posterior end as a result of the forward streaming of the protoplasm. In succeeding drawings (III, IV, V and VI) the moving granules are added in turn to the fixed spiral, until 6 alone is still moving (VI). It is seen that the speed of movement of a granule is twice as fast as the rate of extension of the series of fixed granules; hence the speed of forward movement of the whole organism is half that of the streaming granules. (See, for comparison, the untwisted caterpillar tractor in *b*.)

At the posterior end, granules belonging to the fixed series are passing successively into the forward stream. The granule which most recently began to move is marked 0; proceeding forward along the fixed series, granules are numbered successively: 1, 2, 3, 4, 5 and 6. It is evident that the fixed series (measuring backwards from granule 6) diminishes at half the speed at which the granules advance. (Compare the position of granule 0 in (III) with its position in (IV). During the time interval between these two drawings the fixed series has diminished posteriorly by one granule, while granule 0 has moved through twice the distance between two adjacent granules.)

Bacteria frequently adhere to the edges of the ribbon, and in such cases it is clear from their movement that the movement of the granules is associated with movement of the surface. A bacterium may stick to the edge along which the granules are moving and will then be carried round the helix to the front end, there becoming motionless until it is once more at the posterior end, when it is again carried forward. Such particles do not appear to be ingested.

The sequence of events described here is shown in plates 5 and 6. It must be remembered that it is much more difficult to follow movement such as this in a succession of isolated photographs than in the projected film. The positives in plates 5 and 6 show portions of the cycle of granule movement in two individuals. Figure 3, plate 5 shows particularly clearly the shape and proportions of the organism. It can be seen in the first place that the position of the coils relative to the background does not change; the coil at

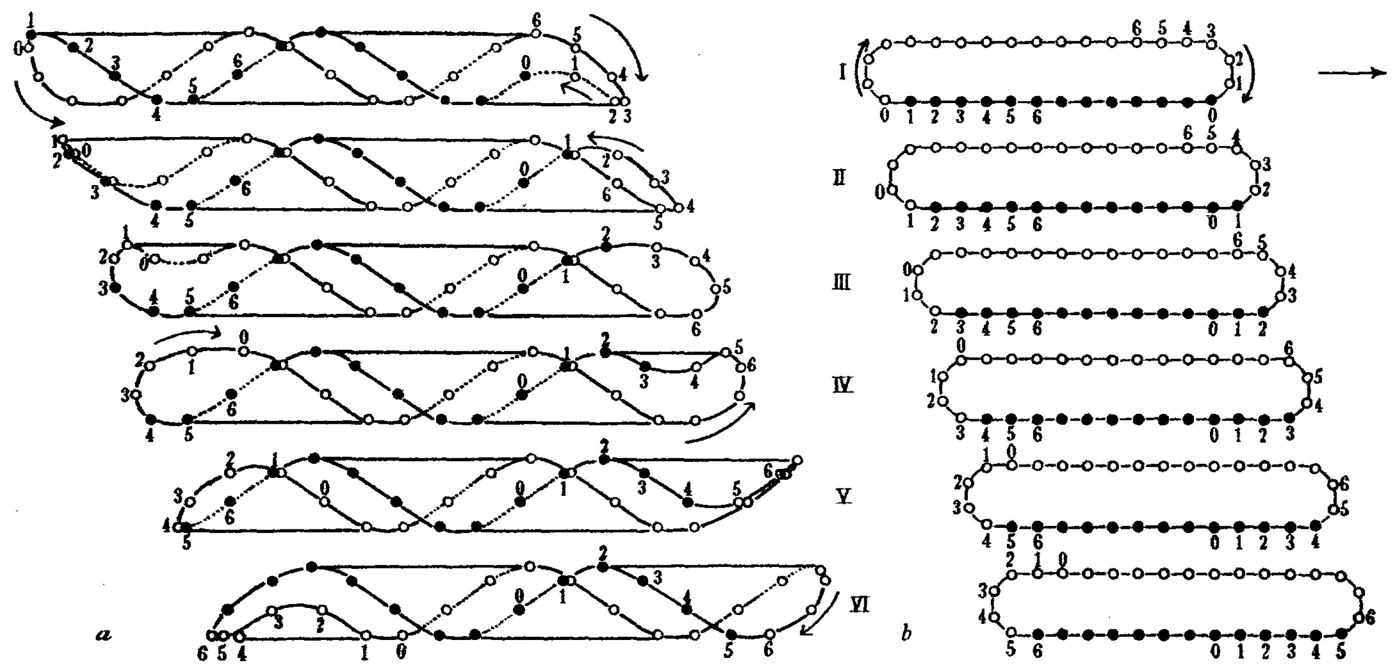

FIGURE 2. The diagram *a* shows six stages (I–VI) in the motion of the helical organism; *b* represents the motion of a caterpillar tractor with the same number of treads (granules) and is obtained by unwinding the helix in *a*. The scale of *b* is half that of *a*. Granules which are stationary relative to the background are represented by black circles, those in motion by white circles. A detailed description will be found in the text (p. 81).

the anterior end in figure 3*a*, plate 5, is still touching the grid in the same manner in figure 3*l*. Starting at the posterior end it is seen that the granule, indicated by an arrow, moves forward along the anterior edge of the ribbon. The rate of forward movement of the granule is greater than the rate of advance of the anterior end; between figures 3*a* and *k*, plate 5, the granule has advanced to the middle of the body while the anterior end has progressed by one quarter to one third of the body length. A stationary granule, *X*, on the posterior edge of the ribbon is seen in figures 3*e*–*i*.

The organism photographed in plate 6 is considerably larger and shows the refractive granules particularly clearly. In figures 5*a* and *b*, plate 6, a particle clearly visible on the posterior edge of the ribbon is stationary while the posterior end advances. In figure 5*c* the previously stationary particle begins to move forward and, changing its position vertically as well as in the plane of the paper, is seen as a black spot (owing to the shift in focus). It emerges from beneath the helix in figure 5*e* and travels forward and along the edge, passing beneath the ribbon in figures 5*h* and 5*i*. This process is repeated twice in the series of photographs shown here. By that time the organism has advanced through a distance of about half its own length, the granule having travelled through about twice that distance.

If, as frequently happens, the organism reverses the direction of movement, the condition of the granules on the two edges is simply reversed: those which were moving become stationary, those which were stationary begin to move in the opposite direction.

It is clear that if the organism is to progress it must be in frictional contact with some surface. Movement of the type described here was always observed with the organism adhering to the cover-slip, or to the slide, or to the surface of algal debris. If the drop was examined immediately after putting on the cover-slip, individuals were occasionally observed floating freely in the liquid. Under these conditions they were never observed to swim like typical spirochaetes. While it is necessary that the organism should be in contact with some surface, it is also clear that there must be relatively little skin friction between the streaming edge of the ribbon and the substratum or the surrounding liquid, otherwise the forward streaming would lead to a backward displacement of the organism. From the shape of the organism it is evident that the fixed series of granules can only be in contact with the substratum at certain points. A greater stickiness of the fixed edge as compared with the moving edge would be entirely compatible with the suggestion, made below, that the streaming edge is in the sol condition, while the fixed edge is gelated.

4. A COMPARISON WITH AMOEBOID MOVEMENT

By analogy with current views on the nature of amoeboid movement (see, in particular, Pantin 1923) we may regard the cycle of changes in the behaviour of a granule as evidence of the cyclical conversion of the protoplasm from sol to gel and from gel to sol. The edge on which the granules are streaming forward may be regarded as in the sol condition. At the end directed forward in movement, streaming protoplasm is being gelated; at the posterior end, gelated protoplasm is passing into the sol condition and streaming forward. How much of the protoplasm is in the sol condition cannot be determined, since the only particles whose movement might indicate the condition of the protoplasm are distributed along the edges of the ribbon. While it is just conceivable that the organism contains a relatively solid, helical core which is displaced forward by the moving edge, just as is the body of a tractor, it seems more likely that there should be a uniform velocity gradient between the two edges, a gradual transition from sol to gel, such as is observed between endoplasm and ectoplasm in an amoeba.

Recent theories of amoeboid movement (see Pantin 1923; Mast 1926) suppose that the formation of a pseudopodium depends on the pressure exerted by the contracting, gelated tube of ectoplasm; pressure is continuously exerted and leads to a continuous squeezing forward of fluid endoplasm, which is then laid down as a gel, extending the contracting ectoplasmic tube forwards. Posteriorly the gel liquefies to form fluid endoplasm. In the case of the green helical organism, there is no apparent contraction leading to the forward streaming of the protoplasm. This corresponds to the condition in the Foraminifera and Heliozoa, where pseudopodia are formed apparently in the absence of any such hydrostatic mechanism, by a process of "micellar crystallization" (see Schmidt 1937*a* and the discussion in Picken 1940). The processes fundamental to amoeboid movement may perhaps be stated as follows: (1) sol ⇌ gel conversion; (2) extension of the gelated ectoplasmic tube in the direction of movement; (3) contraction of the gelated ectoplasm, detachment from the substratum and return to the sol condition. The helical organism exhibits but two aspects of the phenomenon—the cyclical change, sol ⇌ gel, and the extension forward of the gelated helix.

The forward streaming of the granules in the helical organism recalls the forward streaming of the surface film in amoebae. It will be remembered that Jennings (1904) claimed that the entire ectoplasm of forms such as *Amoeba verrucosa* streams forward over the front end of the animal, so that granules lying in the ectoplasm are carried forward on the upper surface, over the advancing anterior end to the ventral surface, and eventually pass to the upper surface once more as the amoeba moves on. Jennings states that the speed of the forward movement of the ectoplasmic granules is about the same as that of the endoplasmic stream. Schaeffer (1920) points out that such rolling movement as Jennings describes is mechanically impossible, unless the granules in the ectoplasm move forward exactly twice as fast as the anterior end. He also shows that the ectoplasm itself is stationary relative to the background, and that the advancing granules are moving in a thin surface film, not in the ectoplasm. This film he compares to a liquid surface. His observations show that the surface of an advancing pseudopodium is moving forward at a rate varying from one to three times the speed at which the tip of the pseudopodium advances. According to Schaeffer the film is formed at the posterior end of the amoeba and is taken into the interior at the tip of the advancing pseudopodium.

Schaeffer's observations provide no support for Jennings's theory of rolling amoeboid movement. The mechanism postulated by Jennings appears, however, to be realized in the green helical organism. Since the relation between forward streaming of the surface film and amoeboid movement is still completely obscure, it is not desired to do more than indicate the resemblance between the rolling movement hypothesis of Jennings and the actual movement of the green helical organism.

5. THE MOTION OF TRUE SPIROCHAETES

The only spirochaetes which have as yet been examined for comparison with the green helical organism are free-living forms: *Spirochaeta fulgurans* Dobell (?) and *S. plicatilis* Ehrenberg. It seems very probable that the movements of these organisms, when in contact with a solid surface, are fundamentally similar to those of the green helical organism. If the more central coils are watched in an individual, the ends of which are extending now in one direction now in the other, they are seen to be completely motionless. There is in this case also, therefore, no rotation of the helix; one end grows as the opposite end diminishes. No granules, however, are visible, the movement of which might give a clue to the fundamental process. If we are to account for the displacement of such a spirochaete when freely suspended in water, it is evident that the friction between the stationary parts and the surrounding liquid must be such that the resistance to backward displacement is greater than that to forward extension of the tip of the helix. It is possible that the forward stream is internal, as in the case of an amoeba of the *limax* type, but the same effect might be obtained if a very small area only (a narrow strip) of the surface were moving, or if for some reason the skin friction between this moving surface and the water were low. It is hoped to study the movements of *Cristispira* and other spirochaetes in order to ascertain how far the movement of the green helical organism described here provides the key to the movement of spirochaetes in general.

It must, however, be realized at this point that there are serious practical difficulties to be overcome in deciding between various possible types of movement. One can imagine a rod-shaped, flexible organism propelling itself through a viscous medium by throwing its body into waves in three dimensions; it takes on the form of a helix, the coils of which will appear to travel backwards from the anterior end. In the case of a macroscopic organism the coils will move slowly backwards relative to the background over which it is moving, since there will be a significant amount of slip between the surface of the organism and the viscous medium. If the organism is of microscopic dimensions, however, it is quite possible that such slipping will be inconsiderable, so that the wave travelling down the organism will be stationary relative to the medium. In films of *Cristispira* (made by Dr J. E. Harris of this department) this appears to be the case; the spirochaete is displaced, as it were, along an imaginary extension of its own helix: the waves are stationary. Given cinematographic records, it would not, therefore, be possible to distinguish by inspection between swimming by means of rhythmic waves of contraction in three dimensions, and "swimming" as a result of forward extension of the helix by some process similar to that observed in the green helical organism, where also the coils of the helix remain stationary relative to the background.

There are two difficulties which have to be faced if we assume that fast-moving spirochaetes propel themselves through the water by waves of contraction. The first arises from the observation, recorded on p. 85, that their movement when in contact with a solid surface resembles that of the helical organism, inasmuch as the more central coils remain stationary relative to the background. The second is the fact that a spirochaete is a helix even when at rest, even after fixation; that is, the *shape* of the organism is helical. It is therefore necessary to suppose that the contraction wave may cease to be propagated and become stationary (relative to the anterior end) at certain times. If this is the case, however, it is somewhat surprising that a spirochaete never loses its wave altogether, but gives rather the impression of being a permanent helix. It is difficult to see how a solenoid-like body could be caused to rotate rapidly about its long axis (as spirochaetes are said to do) unless it were provided with cilia or flagella (like *Spirillum* or *Vibrio*). The slow rotation of the trichomes of certain Cyanophyceae—*Oscillatoria*, or *Spirulina versicolor* Cohn (see Dobell 1912)—may or may not be due to spiral waves of mucous secretion, but it is difficult to imagine such a process leading to the rapid movement observed in spirochaetes.

It may be objected that the speed of movement in *Cristispira* and fast-moving spirochaetes in general is too great for displacement to be brought about by streaming. Films of *Cristispira* sp. from the Oyster, made by Dr J. E. Harris, showed that this spirochaete moves through its own length in 1–3 sec.; but although a speed of 50–100μ/sec. in *Cristispira* is of a very different order from that in the green helical organism, 1–2μ/sec., the movement of *Cristispira* (as I am informed by Dr Harris) would not in fact necessitate a speed of protoplasmic streaming greater than that observed in filopodia of Foraminifera, for example.

It is impossible to decide what is happening by direct or indirect observation of the movement alone. As Nägeli and Schwendener (1877) pointed out, any helix moving in the direction of and rotating about its axis appears to execute snake-like movements, and we have seen that cinematography is unlikely to yield the key to the motion of spirochaetes. Perhaps the most hopeful line of attack would be to look for displacement of granules in a large form such as *Spirochaeta plicatilis* Ehrenberg when creeping on a solid surface, if necessary under dark ground illumination.

6. THE FINE STRUCTURE OF THE GREEN HELICAL ORGANISM

It was observed that the width of specimens diminished during fixation and dehydration, while the length remained practically unchanged. Such anisotropic changes in dimensions are typical of any fibrous micellar aggregate, in which anisodiametric particles are arranged with their long axes parallel to the long axis of the fibril. If water or other liquids can penetrate between the particles, the object will swell or shrink more in width than in length when liquid penetrates to, or is removed from, the intermicellar or intermolecular spaces.

Experiments showed that the shrinkage is not produced by fixation, in the case of fixatives containing osmium tetroxide, but appears during dehydration. The width and length of specimens were determined in life, using a *camera lucida*. The organism was then fixed by bringing a drop of Champy's fluid to the edge of the cover-slip, and measurements were made once more. Absolute alcohol was then added and the specimen well irrigated until no further change in shape occurred. The results of such an experiment are shown in table 1. From this it is clear that the shrinkage in width may reach almost 50 %, that in length, however, barely 5 %. The change in the ratio of width to length is clear from the photomicrograph of fixed, stained and dehydrated specimens shown in figure 4, plate 5.

From these experiments it may be concluded that the organism has a regular, fibre-like structure. Bělař (1929) reached similar conclusions about the structure of mitotic spindles from observations on their anisotropic shrinkage. His conclusions have since been supported by Schmidt's observations (1937*b*) on the birefringence of the spindle. Attempts to detect birefringence in the green spiral organism were not successful, nor did gold-impregnated preparations (made as described by Schmidt 1935) show any trace of dichroism. These negative results do not, however, contradict the conclusions drawn from anisotropic shrinkage. The culture died off so rapidly that any extended experiments (with more powerful light-source, or modified conditions of impregnation) were impossible.

TABLE 1. CHANGE IN DIMENSIONS ON FIXATION AND DEHYDRATION

Measurements of one individual expressed in arbitrary units

	Length	Width
Living	9, 8·5, 9, 8, 8, 10, 9, 8, 9, 9·5 Average 8·8	1·2, 1·0, 1·2, 1·0, 1·1, 1·2, 1·0, 1·1, 1·2, 1·4 Average 1·2
Fixed with Champy's fluid	—	1·1, 1·0, 1·0, 0·9, 1·1, 1·2, 1·2, 0·9, 1·1, 1·2, 1·0 Average 1·1
Dehydrated with absolute alcohol	8·5, 8·0, 8·5, 9·0, 9·0, 8·5, 8·0, 8·0, 8·0, 8·0 Average 8·4	0·5, 0·7, 0·7, 0·6, 0·5, 0·5, 0·5, 0·7, 0·7 Average 0·6
Shrinkage	5 %	50 %

In attempting to detect dichroism, a difficulty was encountered which appears insuperable. The blue-yellow cross (a subjective effect produced by plane polarized light) made it impossible to decide the colour of any minute object lying at the centre of the cross. (The cross is always visible in that part of the field towards which vision is directed.) Since either the cross rotates on rotating the polarizer, or the object must be rotated if the polarizer is kept stationary, the colour change in the background from blue to yellow or yellow to blue tends to induce a contrast change in the colour of the object such that it would be easy to imagine that a dichroic effect exists.

If we now attempt to put together what has been learned of the structure of the organism and what was learned from its movement, we can perhaps best picture the creature as a skein of wool, twisted into a screw. The threads of wool represent the anisodiametric particles, which are arranged almost parallel to its long axis. Many of the threads are tied up to form parallel bundles—representing the gelated portion—while a band of threads is left free among one edge—representing the streaming protoplasm in the sol condition. At the anterior end we may assume that molecules or micells from the sol phase are added to the gel structure according to the helical plan. It is simplest to suppose that, in being pulled into position to be added to the gelated helix, the region of protoplasm associated with one granule pulls on the next portion of the liquid band and so brings it into position. In the model this is represented by the continuity of the threads, but we need not assume that the particles of the organism form a continuous skein.

The helical organism forms, as it were, part of an imaginary helix extending in either direction beyond the limits of the body; at any moment part of this helix is realized. The form of the organism is constant—it is always a right-handed helix of so many turns—but its projection on a given plane, that is, its apparent shape, when viewed from a given point, is continually changing (see figure 2). We might say that the green helical organism moves by growing, in the sense in which we speak of the growth of a pseudopodium. Growth in the more profound sense of the manufacture of substance to be built up according to the helical plan leads to an increase in length of the organism, but not to any considerable increase in width (see figure 1 and compare figure 3, plate 5 with figure 5, plate 6). This means that new protoplasmic material tends to be added to the structure in the same way as the existing material is added to the anterior end in the course of movement. From a morphological point of view the organism appears in this case to be built up by the repetition of a pattern in space, in a manner analogous (and only *analogous*) to that in which a crystal is built up. When we consider the activity of the organism, however, it is clear that we are dealing with a kind of pattern fundamentally different from that of the crystal.

The continuous renewal of the helical organism in accordance with a predetermined pattern is the result of differences in activity between one part of the organism and another. There is in the first place an anteroposterior polarity. Dobell (1912) and, following him, Hindle (1931) suggest that spirochaetes possess no polarity, since they move indifferently in either direction parallel to their long axis. It is of course true that their polarity is not fixed, but, on the other hand, in the case of the green helical organism, there exists at any moment (other than that at which the organism reverses its direction of movement) a well-defined physiological and, ultimately, morphological polarity. At the anterior end protoplasm is passing from the sol to the gel condition; at the posterior end from gel to sol. There is also a difference in activity between the two edges of the ribbon; the anterior edge is streaming, the posterior edge stationary. It is also conceivable that the inner and outer faces of the ribbon are not in the same condition. It has been shown (see Frey-Wyssling 1935) that the helical coiling of the elaters of *Equisetum* is related to their micellar structure and to differential hydration of the two surfaces. The helical form of the green organism may, by analogy with the elater, result from an actively maintained state of differential hydration of the two surfaces. This view is supported by the observation that the pitch of the helix increases when the organism is dehydrated.

These points all suggest that the structural pattern is inseparable from a pattern of activity. We have a glimpse of how closely the two may be related: the antero-posterior polarity is parallel to the "fibre axis" of the organism, that is, to the axis of molecular or micellar orientation, and this is the direction in which the organism builds itself anew in moving, and in which it also grows.

7. SYSTEMATIC POSITION

Until occasion arises for further study, the systematic position of this organism must remain undecided. It appears to be closely related to the Spirochaetoidea and to certain Cyanophyceae (*Spirulina*). According to Dobell (1912) the characters common to Spirochaetoidea and *Spirulina* are: "absence of antero-posterior polarity; presence of a spiral, flexible body clothed with a pellicle; absence of organs of locomotion; plasmolysability; transverse division; metachromatic granules; variation in length but not in breadth in individuals of the same species; absence of sexual phenomena. The Cyanophyceae differ from the Spirochaetoidea in possessing chromophyll and cyanophycin, in cytoplasmic and nuclear structure, and in lacking a crista or homologous organ—which, however, is also lacking in many Spirochaetes."

The resemblances between certain Cyanophyceae (such as *Spirulina*) and spirochaetes are, however, "merely those general resemblances which exist between all Bacteria and Cyanophyceae, and which mark the close relations existing between these two groups".

Geitler (1925) states that the differences between *Spirulina* and spirochaetes are: absence of an axial filament; inflexibility; presence of cross walls. These criteria are inadequate since (1) an axial filament appears to be absent in many small spirochaetes; (2) the species of *Spirulina* examined by Dobell, *S. versicolor* Cohn, showed marked flexibility; (3) fixed specimens of the genus *Cristispira* (Spirochaetoidea) show chambered structure while, as Geitler points out, cross walls are either absent or only visible in fixed material in the smaller forms of *Spirulina*, which he places in the subgenus *Euspirulina*.

Geitler asserts that the members of the genus *Spirulina* rotate about the long axis as they progress, but Dobell observed that, although *S. versicolor* Cohn sometimes shows slight rotation about the long axis during gliding or creeping, it does not usually rotate.

If, as Geitler claims, all organisms belonging to the genus *Spirulina* rotate about their long axes as they advance, the helical organism cannot belong to this genus. The descriptions of the smaller forms are, however, extremely inadequate. It is impossible in the present state of our knowledge of these forms to state categorically that the helical organism is not a *Spirulina* and equally impossible to exclude it from the Spirochaetoidea.

Dobell (1912), having pointed out that the Spirochaetoidea have many characters in common with the Bacteria, states that "there is only a single feature in which the Spirochaetes differ from the Bacteria: namely, the Bacteria are motile by means of special organs of locomotion (with the exception of forms such as *Beggiatoa* whose movements resemble those of the Cyanophyceae), whereas no such organs exist in the Spirochaetes".

Hindle (1931) defines the Spirochaetes as "a fairly distinct group of organisms characterized by their spiral form, flexibility and the power of active movement without the aid of flagella or any other organs of locomotion, except possibly in the case of developmental forms which may show a spiral terminal flagellum". He considers them to be most closely related to bacteria such as *Spirillum*, which is not flexible. The largest free-living form, *Spirochaeta plicatilis* Ehrenberg, from fresh-water, reaching a length of 500μ and a width of $0{\cdot}75\mu$ (Zuelzer 1910), differs from the green helical organism in the presence of an axial filament running down the centre of the closely wound helix, and of volutin granules disposed on either side of the axial filament (see figures in Hindle 1931). No preparation of the green helical organism shows any trace of an axial filament. Unhydrolysed Feulgen preparations, made according to Reichenow's procedure (see Doflein-Reichenow 1928), showed no trace of volutin granules, nor indeed did any balsam preparation show the granules. These characters do not however enter into the definition of the group, and they are not recognizable in the smaller free-living or pathogenic forms. How closely the green helical organism is related to the Spirochaetoidea would seem to depend on how far the type of movement described is characteristic of spirochaetes.

Although a parallel with amoeboid movement has been drawn, this does not imply any close relationship between this organism and the Sarcodina. It is of importance to decide whether *Cristispira* moves in the same way as the green helical organism, since fundamental differences in the type of movement may well justify a separation of existing spirochaetes by more than generic differences. If the green helical organism divides by transverse fission, as the evidence suggests, it should be regarded on Hindle's definition as a spirochaete. On the other hand, it seems fairly certain that the organism described here is not Lagerheim's green spirochaete, *Glaucospira* (1892), which Geitler, with due caution, includes in the genus *Spirulina*. Lagerheim states that the threads normally swam at high speed, thrown into a loose secondary spiral (as does *S. fulgurans* Dobell), but would on occasion suddenly change their type of movement and show violent contortions in all directions, "wie eine verwundete Schlange". He does not doubt that cilia are present at the ends. It is clear that this account could not possibly apply to the organism described in this paper. Nor for that matter do the descriptions of *Glaucospira*-like forms given by Doflein (1911) suggest that he is describing our form. On this account Lagerheim's genus will not be revived to include the new helical organism. Warming (1875) described a very large spirochaete, *Spirochaeta gigantea*, but it is evident that his description cannot refer to the green helical organism. The systematic position of the latter must remain undecided until the opportunity occurs of examining it once more, and until more is known of the mechanism of movement in other spirochaetes.

The cinematographic films of the green helical organism were made in collaboration with Mr K. C. Williamson, to whom I am greatly indebted. I wish to acknowledge my debt to Mr C. Dobell, F.R.S., Professor J. Gray, F.R.S., Professor E. G. Pringsheim and Dr D. A. Webb for many helpful suggestions. My special thanks are due to Dr J. E. Harris, who has allowed me to examine his films and to quote from his unpublished results.

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DESCRIPTION OF PLATES 5, 6

All photographs were taken with a 2 mm. apochromatic objective (n.a. 1·32) and a × 20 periplanatic eyepiece. The final magnifications are given at the end of each legend.

The effect of field size and pattern on the change of visual sensitivity with time

By B. H. Crawford, M.Sc.

(*Communicated by Sir John Parsons, F.R.S.—Received* 29 *March* 1940)

Results are recorded showing the effect of size of a circular conditioning field on brightness threshold measurements made at the centre of the field: first, with the field steadily exposed and viewed; secondly, when the conditioning field has been cut off and the eye is returning to a state of dark adaptation. Conditioning and test fields are both illuminated by white light. No field size effect is detectable over the range 60° diameter down to 3° diameter. Below 3°, for a steady state of adaptation, various rather complex effects of interference between the conditioning fields are shown; these are explained in terms of the effect of distant parts of the conditioning field in raising the threshold and a perceptual interference between the patterns of conditioning and test fields. For changing states of adaptation the effect of field size cancels out—except in an isolated case—if the initial conditioning field brightnesses are adjusted to give the same steady value of threshold: then, after cutting off these various conditioning fields, the decrease of brightness threshold follows the same course. However the test patch of retina be brought to a given steady state of adaptation, as measured by the brightness threshold, it will recover its dark adaptation in the same way. These considerations apply both to foveal vision and to vision in the parafovea at 8° from the fovea.

1. Introduction

In pursuance of work on the change of visual sensitivity with time (Crawford 1937*a*) investigation has been made of the effect of the size of the initial conditioning field on the course of the change of visual sensitivity after cutting off the conditioning field. As an adjunct to this work it was also necessary to determine the effect of size of conditioning field on sensitivity measurements made with the conditioning field steadily exposed and the eye in equilibrium with it; the results obtained on this equilibrium effect are also given. The measure of sensitivity used, as in the earlier work, is the threshold brightness of a small test field; sensitivity may be regarded as the inverse of the threshold brightness.

The question of size of the conditioning field is one aspect of the general question of the pattern of conditioning field presented to the eye. In this paper the size is varied from very large compared with the test field to smaller than the test field; in all cases the two fields are circular and concentric. Another pattern of conditioning field was also used, namely a ring with dark central portion, the test field appearing at the centre of the ring, as it was thought that such a pattern might possibly show up some form of conduction of the condition of adaptation across the retina. The results were negative, but are briefly given.

2. Apparatus

As the pupil size varies with the size of the illuminated part of the visual field (Crawford 1936), Maxwellian view of the conditioning and test fields was used, the cross section of the beams as they passed into the eye being less than the smallest possible size of the eye pupil. In order to investigate a wide range of sizes of conditioning field, two arrangements of apparatus were necessary, shown in figures 1 and 2. The arrangement shown in figure 1 is essentially the same as that given in a previous paper (Crawford 1937*b*), with the addition of a series of diaphragms which can be placed at *H* to limit the size of the conditioning field, the range of sizes being from zero up to about 12°. These diaphragms are carried by a mechanical microscope stage to facilitate centering with the test field. The fixation spot is also arranged more simply than before, being a small lamp *V* at a distance of some 3 m. from the apparatus, seen by reflection in the plain glass mirror *W*. This mirror is slightly wedge-shaped, so that two separated images of *V* are seen. For foveal vision these two images are

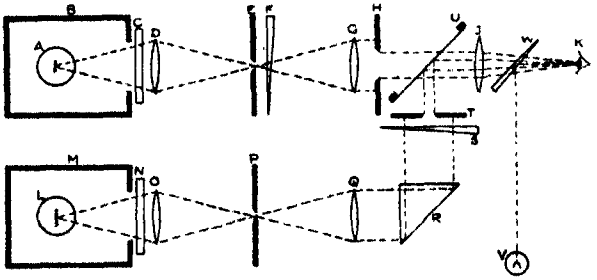

FIGURE 1. Plan of apparatus for small conditioning fields. *A*, lamp for illumination of conditioning field; *B*, light-tight box; *C*, neutral filter; *D*, lens forming image of *A* at *E*, aperture and shutter; *F*, neutral wedge; *G*, lens rendering beam of light parallel; *H*, aperture to limit size of conditioning field; *J*, lens converging beam to a focus in centre of pupil of subject's eye, *K*; *L*, lamp for test field illumination; *M*, light-tight box; *N*, neutral filter; *O*, lens forming image of *L* at *P*, aperture and shutter; *Q*, lens rendering beam of light parallel; *R*, reflecting prism; *S*, neutral wedge; *T*, aperture to limit size of test field; *U*, pellicule mirror (thin film of celluloid); *V*, lamp for fixation point (3 m. from apparatus); *W*, wedge-shaped plain glass mirror.

arranged to appear symmetrically on opposite sides of the test field, for parafoveal vision one only is used at the requisite separation from the test field.

For a size of Maxwellian field greater than about 15° a somewhat complex lens system would be necessary; a concave mirror system arranged as in figure 2 was therefore chosen. This gives a field size of 60° without trouble. The eye pupil *M* and the source of light *N* were both placed as close to the centre of curvature of the mirror *O* as possible, to reduce aberrations in the image formed at the eye pupil. It is only necessary that these aberrations should be small enough to allow the whole image to fall within the limits of the pupil, a condition which can just be satisfied with a mirror of about 14 cm. diameter and the same radius of curvature. The test spot beam is the same in principle as in figure 1. Limitation of the conditioning field size was by means of apertures such as *P*. It is not possible to use very small apertures, comparable with the test field size, because they are seen somewhat out of focus; conditions would therefore be ill-defined for small field sizes, and so the two arrangements of apparatus are necessary.

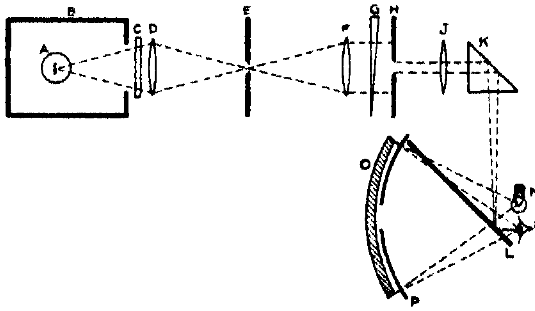

FIGURE 2. Plan of apparatus for large conditioning fields. *A*, lamp for test field illumination; *B*, light-tight box; *C*, neutral filter; *D*, lens forming image of *A* at *E*, aperture and shutter; *F*, lens rendering beam parallel; *G*, neutral wedge; *H*, aperture to limit size of test field; *J*, lens converging beam to a focus in centre of pupil of subject's eye, *M*; *K*, reflecting prism; *L*, plain glass mirror; *N*, lamp for illumination of conditioning field; *O*, concave spherical mirror; *P*, aperture to limit size of conditioning field.

It may be mentioned here that field brightnesses are specified by measuring the candle power of the final image at the eye position and expressing the result, by calculation, as a brightness seen through a circular pupil of 3 mm. diameter. The necessary measurements are easily carried out by a Maxwellian view photometer or illuminometer, observing the conditions of observation laid down by Collier and Taylor (1938).

3. RESULTS

3·1. *Steady state of adaptation*

The results for steady states of adaptation are described first, as some of them are needed to define the conditions of experiment for changing states of adaptation. Also, the conditions of experiment are simpler and it is therefore natural to start with them. Measurements of brightness threshold were made after the eye had come to sensible equilibrium with a steady conditioning field for various sizes and brightnesses of the latter. Two retinal areas were taken, one at the fovea, the other in the parafovea, its position being defined by the test field at a distance of 8° to the nasal side of the fixation spot. This particular parafoveal position was chosen as being fairly representative of the parafoveal area in general and far away from the discontinuity of the blind spot. The results are shown in tables 1 and 2, and figures 3–6. In all cases the readings are for the author's right eye, with white light in both conditioning and test fields.

TABLE 1

Retinal area tested: fovea. Test field diameter: 0·45°. Test field exposure: 1 sec.

Conditioning field		Test field: mean threshold brightness c./ft.^{2}
Brightness c./ft.^{2}	Diameter	
710	60°	4·0
710	9°	4·1
69	60°	0·51
69	9°	0·51
4·7	60°	0·0209
4·7	9°	0·0224

TABLE 2

Retinal area tested: 8° from fovea. Test field diameter: 0·45°. Test field exposure: 1 sec.

Conditioning field		Test field: mean threshold brightness c./ft.^{2}
Brightness c./ft.^{2}	Diameter	
4·7	60°	0·048
4·7	9°	0·050
0·0001	60°	0·000110
0·0001	9°	0·000120

From tables 1 and 2 it appears that the threshold for a 9° conditioning field is either equal to or greater than that for a 60° field, though the difference is small, the mean difference being 5 %. This is less than the probable error of a threshold reading (about 7 % under most conditions) and it is, therefore, doubtful whether it is a significant difference. It may be noted that it tends to be greater at low brightness levels.

In view of this coincidence of results for the two field sizes it was not considered necessary to use more than one subject. The same consideration applies to most of the subsequent results. It may be added that the author's eyes are normal in every way and in various past comparisons with other eyes for visual capacities have been found to lie near the average.

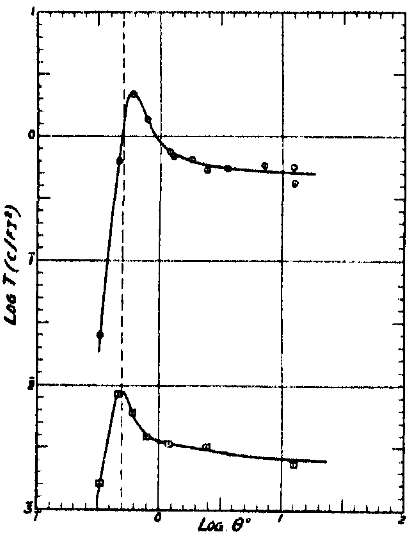

FIGURE 3

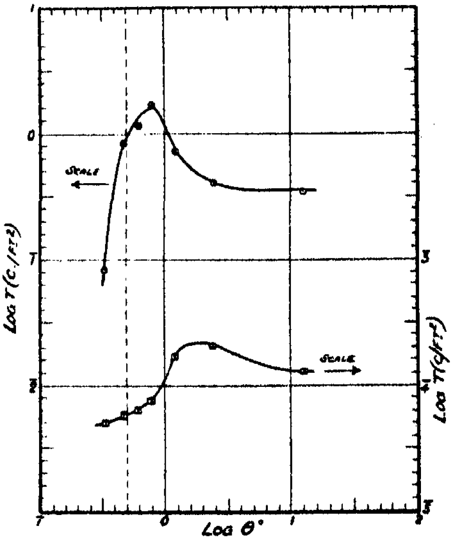

FIGURE 4

FIGURE 3. Variation of brightness threshold (T) with diameter of conditioning field (θ). Subject B. H. C. Fovea. Test field diameter, 0·50°; exposure, 1 sec. ⊙, conditioning field brightness = 39 c./ft.^{2}. □, conditioning field brightness = 0·19 c./ft.^{2}.

FIGURE 4. Variation of brightness threshold (T) with diameter of conditioning field (θ). Subject B. H. C. Parafovea 8°. Test field diameter, 0·50°; exposure, 1 sec. ⊙, conditioning field brightness = 3·8 c./ft.^{2}. □, conditioning field brightness = 0·000123 c./ft.^{2}.

Further results for smaller conditioning fields, some actually smaller than the test field, are given in figures 3–6. In figures 3 and 4 data are plotted showing the variation of threshold with conditioning field size for various fixed values of the brightness of the latter field. Figure 3 refers to foveal vision, figure 4 to parafoveal vision. In all cases the same general features are shown; starting from the larger diameter fields the threshold is at first constant—which one would, of course, expect from the com-

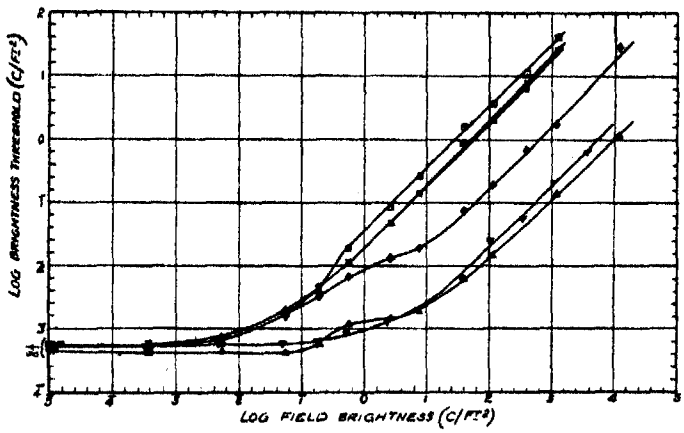

FIGURE 5. Variation of brightness threshold with brightness and size of conditioning field. Subject B. H. C. Fovea. Test field diameter, 0·49°; exposure, 1 sec. ×, conditioning field diameter of 10·3°; ⊙, 3·06°; □, 1·09°; ◇, 0·33°; △, 0·14°; ▽, ring-shaped conditioning field, outer diameter 12°, inner diameter 4·9°.

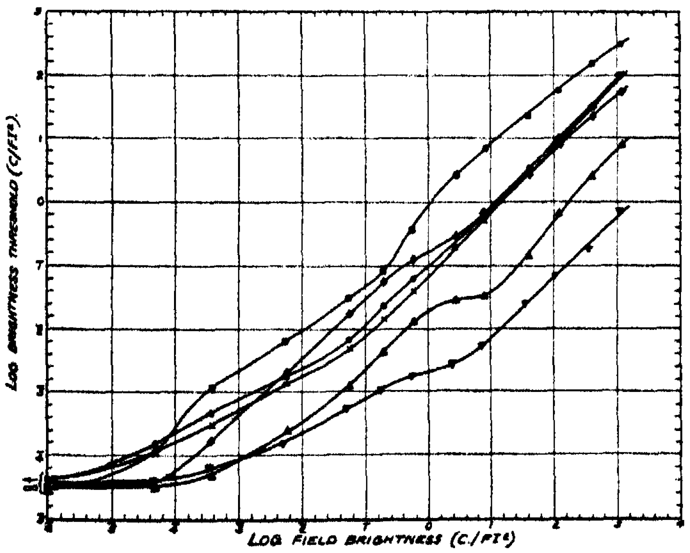

FIGURE 6. Variation of brightness threshold with brightness and size of conditioning field. Subject B. H. C. Parafovea 8°. Test field diameter, 0·49°; exposure, 1 sec. ×, conditioning field diameter of 10·3°; ⊙, 3·06°; □, 1·09°; ◇, 0·33°; △, 0·14°; ▽, ring-shaped conditioning field, outer diameter 12°, inner diameter 4·9°.

parisons of tables 1 and 2—it then rises to a maximum and subsequently falls away more or less rapidly. There are, however, interesting variations in detail. Consider the position of the maximum in the various cases. In the two foveal curves, figure 3, the maximum is very near that value of conditioning field size which is equal to the test field size. Indeed, the data do not definitely show that the maximum is not actually at this point. In the two parafoveal curves, on the other hand, the maxima are definitely displaced to larger values of conditioning field size, and the lower the brightness level the greater is the displacement.

The suggested explanation of this effect involves the supposition that the conditioning field can raise the threshold brightness of the test field in two ways. In the first place, the threshold is raised by virtue of the brightness of the conditioning field, which may be effective at a distance from the test field boundary (an integrative effect). If this integrative effect were present, removal of the outer zones of a conditioning field larger than the test field would allow the threshold to drop, and for a large integrative effect the threshold would begin to drop with decreasing size of conditioning field when the latter was comparatively big compared with the test field. In the second place, the pattern of the conditioning field may interfere with discrimination of the pattern of the test field, and such interference will also raise the threshold. In the above experiments this pattern interference would be a maximum when conditioning and test fields are the same size. Applying these ideas to the curves of figures 3 and 4 it would appear that at the fovea there is no appreciable integrative effect, for the maximum of the curve is at the point where the conditioning field is equal in size to the test field: the pattern interference appears alone and unmixed with any other effect. In the parafovea, however, there appears to be a very appreciable amount of integrative effect, for the maxima of the curves are displaced more or less from the point where conditioning and test fields are equal in size, in the direction of larger values: the maximum, its height and its position are a resultant of integrative effect and pattern interference. It may be noted that the integrative effect is greater at low brightness levels. The absence of integrative effect in the fovea (except, possibly, for very small fields, which are not considered here) and its presence in the parafovea, especially at the lower brightness levels, are all in accord with what is already known from measurement of brightness threshold for different sizes of test field.

In figure 5, relating to foveal vision, and figure 6, relating to parafoveal vision, the influence of field size on brightness threshold is further exemplified in the form of curves of brightness threshold plotted against conditioning field brightness for various sizes of the latter field. Results for a ring-shaped field are also included. These curves were obtained primarily in order to determine, for each field size, the brightness which gave a certain fixed value of threshold, but they present features of intrinsic interest also.

Considering foveal vision, figure 5, it will be seen, in the first place, that all the curves eventually become linear with a slope of very nearly unity, that is, the Weber-Fechner law is obeyed for all sizes of conditioning field above a sufficiently high brightness level. On the other hand, the curves cannot be fitted together along their entire courses by sliding laterally, and hence the equivalent background hypothesis is only of limited applicability, in fact, it can only be applied to the linear portions of the curves.

The curves for field diameters of 10·3° and 3·06° are virtually coincident, showing that field size has little influence on foveal measurements down to about 3°, assuming, of course, that the test field is 0·5° or less. Below 3° the curves begin to show signs of breaking up into two portions, rather similar to the two portions of the parafoveal threshold curve. But in this case the cause is probably different; it seems likely that it is a change in form perception, occurring at some brightness level between 0·3 and 0·6 c./ft.^{2}.

In the case of parafoveal vision, figure 6, things are, as might be expected, a little more complex, since there are two visual mechanisms involved (photopic and scotopic) besides this possible change in form perception. The linearity of the curves with slope of unity at higher brightness levels is no more than approximate, except for conditioning field sizes of 10·3° and 3·06°. This may be due to the patterns of the smaller fields being too small to be perceived purely as contrasts by the comparatively gross aggregates of percipient elements in the parafovea. One cannot, in consequence, expect the Weber-Fechner law to hold except as a very rough approximation.

Regarding the breaking up of the curves into portions, the parafoveal results also show transitions at values of conditioning field brightness similar to the foveal ones, but rather more widely spread, from 0·2 to 0·9 c./ft.^{2}. This at least suggests that a similar mechanism—change of form perception—is operative in the two cases. The curve for a conditioning field of 1·09° diameter shows also an interesting change of course in the neighbourhood of 0·0001 c./ft.^{2}. It is possible that this is indicative of a change in form perception in the scotopic visual mechanism.

3·2. *Changing state of adaptation*

In this part of the work the eye was first exposed to a conditioning field of a certain size and brightness for a given time, the conditioning field was then cut off and threshold measurements made at intervals afterwards until complete dark adaptation was approached. The full details of the methods of making these measurements have been described in an earlier paper (Crawford 1937*a*). The investigation falls naturally into two parts: the comparison of the effects of large and medium-sized conditioning fields (apparatus of figure 2), and the comparison of the effects of fields varying in size from medium to very small (apparatus of figure 1).

3·21. *Comparison of large and medium sized conditioning fields*

It has already been shown (tables 1 and 2) that under steady conditions of adaptation the same brightness threshold is obtained at the centre of a conditioning field whether it has a diameter of 60° or 9°. The same two sizes have also been compared for their effect on the course of the change of visual sensitivity after cutting off the conditioning field. The results are shown in figure 7, and it may be said that, within the limits of accuracy of this kind of measurement, there is no difference in the effects produced by these two field sizes for foveal or for parafoveal vision.

3·22. *Comparison of medium and small sized conditioning fields*

In this case, under steady conditions of adaptation, the various smaller sizes of conditioning field tested gave quite different values of brightness threshold for the same value of conditioning field brightness. It is therefore more than likely that different curves of change of visual sensitivity would be obtained after cutting off such fields of equal brightness, and the experiment would prove nothing one way or the other. Consequently, it was decided to compare the various sizes of conditioning field after their respective brightnesses had been adjusted to give the same value of brightness threshold under steady conditions. Having chosen a value of brightness threshold these field brightnesses can be read off directly from figures 5 and 6. The value of brightness threshold actually taken was 1 c./ft.^{2} (log brightness threshold = 0), and the values of conditioning field brightness giving this threshold for the various sizes of conditioning field are taken from figures 5 and 6. Figures 8 and 9 present the results of this part of the work. All experimental points plotted are means of three separate determinations, except for the longer times in the parafovea, where individual points are plotted.

A broad general principle emerges at once. When different sizes and forms of conditioning field are compared they produce the same effect on the course of the recovery of visual sensitivity after they are cut off if they produced the same initial or steady value of brightness threshold while they are still exposed. There are some exceptions to this general principle. In the fovea, for a conditioning field of diameter 1·09°, the general rate of recovery of sensitivity is slower than for the field of diameter 10·3° (taken as a standard of comparison). This may be due to the observed fact that this size of field leaves a persistent after image which interferes noticeably with perception of the test field. In the parafovea, figure 9, no interfering

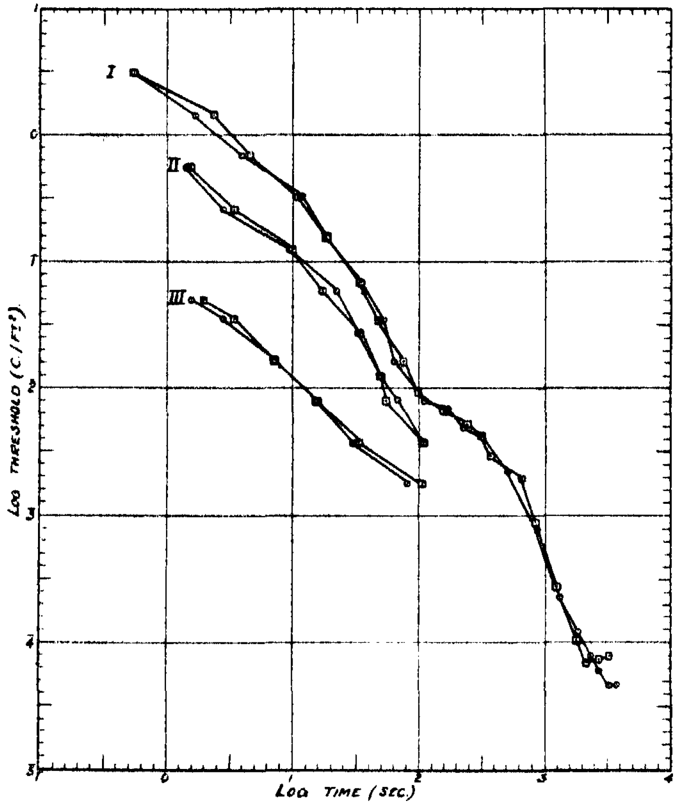

FIGURE 7. Variation of brightness threshold with time after cutting off conditioning field: comparison of large and medium sized fields. Subject B. H. C. Test field diameter, 0·50°; exposure, 1 sec. Conditioning field exposed for 1 min.; ⊙, diameter = 9°; ⊡, diameter = 60°; I, brightness = 440 c./ft.², parafovea 8°; II, brightness = 710 c./ft.², fovea; III, brightness = 69 c./ft.², fovea.

after image was noticed and there is approximate agreement between the recovery curves for 1·09° and 10·3° fields.

It will be noticed that when the conditioning field is smaller than the test field there is considerable irregularity in the points through which

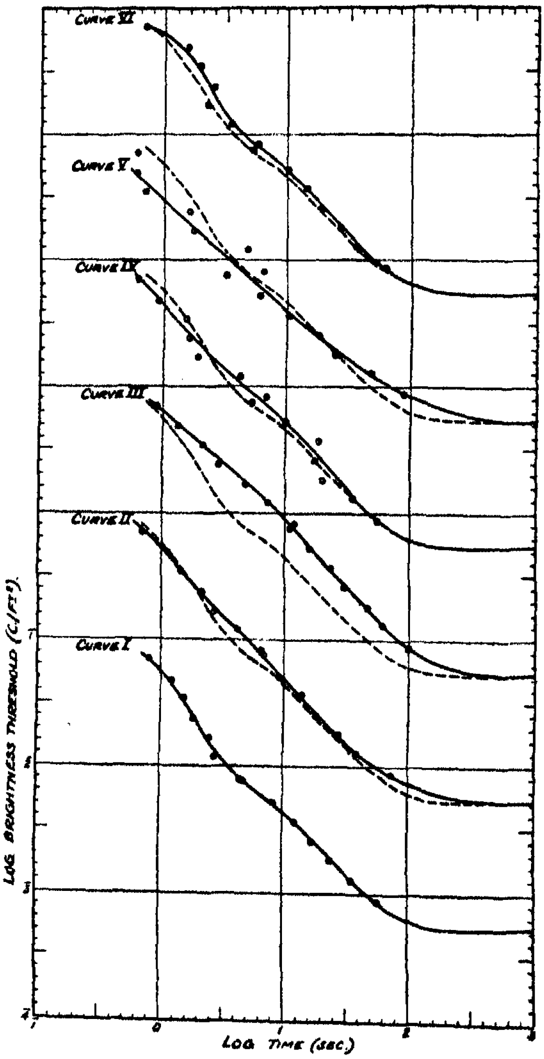

Figure 8. Variation of brightness threshold with time after cutting off conditioning field: comparison of medium and small sized fields. Subject B. H. C. Fovea. Test field diameter, 0·49°; exposure, 1 sec. Curve I, conditioning field diameter of 10·3°; curve II, 3·06°; curve III, 1·09°; curve IV, 0·33°; curve V, 0·14°; curve VI, *ring field*, outer diameter 12°, inner diameter 4·9°. Dotted curve is curve I reproduced for comparison. Each curve shifted vertically by one unit relative to previous curve.

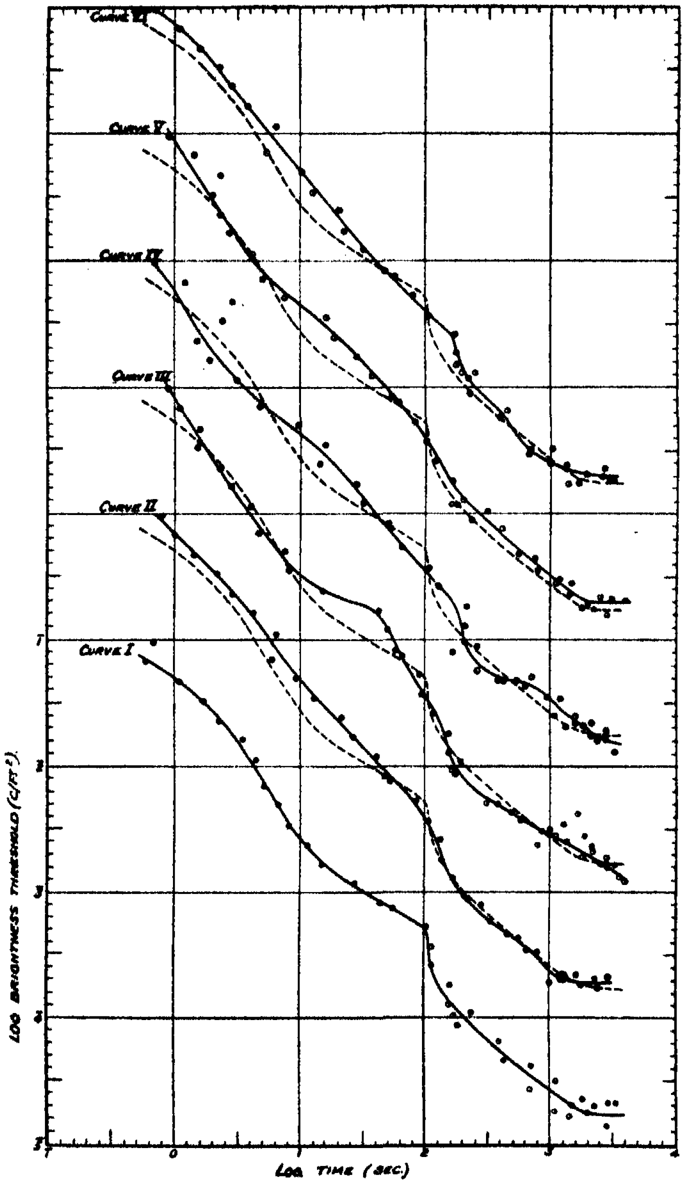

FIGURE 9. Variation of brightness threshold with time after cutting off conditioning field: comparison of medium and small sized fields. Subject B. H. C. Parafovea 8°. Test field diameter, 0·49; exposure, 1 sec. Curve I, conditioning field diameter of 10·3°; curve II, 3·06°; curve III, 1·09°; curve IV, 0·33°; curve V, 0·14°; curve VI, ring field, outer diameter 12°, inner diameter 4·9°. Dotted curve is curve I reproduced for comparison. Each curve is shifted vertically by one unit relative to previous curve.

curves are drawn. This is mainly due to the impossibility of maintaining perfectly steady fixation. Nevertheless, even for these extreme cases, the recovery curves agree within the probable experimental error with those for a conditioning field of 10·3° diameter. Figure 7 demonstrates that they also agree with a field of 60° diameter. Regarding the minor variations in shape of recovery curve, which are especially apparent in the parafovea, it is felt that many of these are probably real, but that any attempt at a detailed explanation of them would at present be premature.

The comparisons of ring and disk conditioning fields may be specially mentioned. Agreement between recovery curves in the two cases is good, especially for foveal vision, and it is obvious that no special elucidation of the possible propagation of adaptation across the retina is to be found from an experiment of this type. The principle remains that, by whatever pattern of conditioning field the retina is brought into equilibrium to have a certain threshold sensitivity, it will recover its dark sensitivity in the same way when the conditioning field is cut off.

Grateful acknowledgements are due to the Illumination Research Council, under whose auspices the work was done.

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Ionic regulation in *Carcinus maenas*

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[Plate 7]

Analyses have been made, with respect to the principal inorganic constituents, of the blood and urine of *Carcinus*, when living in normal sea water, diluted sea water, and sea water modified by the addition of magnesium sulphate.

The composition of the blood of individuals living in normal sea water is as follows (the concentration of each ion being expressed as a percentage of the concentration that would be expected if the blood were in dialysis equilibrium with the external medium): Na 110 %, K 118 %, Ca 108 %, Mg 34 %, Cl 104 %, SO_4 61 %.

This ionic regulation is the resultant of the following processes: active absorption by the gills of sodium, potassium, calcium and chloride at a rate greater than that at which they are lost by diffusion; differential excretion by the antennary gland, which tends to conserve potassium and eliminate magnesium and sulphate; inward diffusion across the gills of magnesium and sulphate in accordance with the concentration gradient.

In normal conditions there is active absorption of water. In dilute media, when osmoregulation begins, this is suspended, and possibly there is a fall in the passive permeability of the gills to water. In other respects osmoregulation is brought about by an intensification of the processes responsible for ionic regulation.

The permeability of the cuticle under physiological conditions is such that it does not affect the salt and water exchange of the animal, which is controlled by the branchial epithelium.

The structure of the gills of four species of Decapoda is described, and correlated with their powers of osmotic and ionic regulation.

INTRODUCTION

The problems of osmotic and ionic regulation have attracted much attention in recent years, especially after the revival of interest in the subject by the work of Duval (1925) and Schlieper (1929 *a*). Among invertebrates interest has centred mainly around the decapod Crustacea. Certain important points have been established with regard to the reactions of several species to variation in the composition of their environment, but the nature of their osmoregulatory mechanisms remains obscure, and the extent of their powers of ionic regulation has not yet been fully described, much less accounted for. Since there are good reasons for believing that osmotic is secondary to ionic regulation (Pantin 1931 *a*), this investigation is primarily concerned with the latter process, but it attempts also to establish the nature of the connection between the two. It was thought that an intensive study of a single species was most likely to yield useful results. The common shore crab, *Carcinus maenas*, was selected, not only for reasons of convenience, but also because its osmotic behaviour has been studied by several authors, especially Fredericq (1904), Duval (1925), Schlieper (1929 *a*), Margaria (1931), Bateman (1933) and Huf (1936), and is by now fairly well known, and because it is the only form other than *Eriocheir* (a much more highly specialized type from the physiological point of view) that is known to have well-developed powers of both osmotic and ionic regulation.

Carcinus maenas is sufficiently euryhaline to be able to live permanently in water of which the chlorinity is as low as 5·5 °/₀₀ (Duval 1925). This is achieved partly by osmoregulation, and partly by ability to tolerate a fall in the concentration of salts in the blood. Duval showed that the blood of Mediterranean specimens, which were kept in dilute sea water of 7 °/₀₀ chlorinity, contained 14 °/₀₀ Cl when equilibration was complete. This is only 65 per cent. of the concentration normally present in the blood of Mediterranean specimens. Schlieper (1929 *a*) found that the blood of crabs living naturally in the dilute waters of the Baltic (Cl = 7 °/₀₀) had also a chlorinity of 14 °/₀₀, as against 18–19 °/₀₀ for those from the North Sea.

THE NORMAL COMPOSITION OF THE BLOOD

Analyses of the blood of *Carcinus* have been made by Bethe (1929), Bethe and Berger (1931) and Bialaszewicz (1933). Their figures, which are set out in table 1, agree in showing that the blood differs markedly in composition from sea water. It has been pointed out (Robertson and Webb 1939; Robertson 1939) that the analytical methods used by these authors leave much to be desired, so that their actual figures need not be taken too seriously. Nevertheless they indicate that the direction in which the composition of the blood of *Carcinus* differs from that of sea water is in a higher content of potassium and a lower content of magnesium and sulphate, so that it falls into line with all other known cases of ionic regulation.

Clearly the first step necessary for an elucidation of the regulatory mechanism was to obtain some more accurate analyses of the blood of individuals living under normal conditions. Most of the crabs were obtained from Millport: a few from Plymouth. At both stations their normal habitat was water of 18–19 °/₀₀ chlorinity. All the individuals used were large males, of 70–120 g. weight, which had neither recently moulted nor were obviously about to moult. Before the blood samples were taken the crabs were kept for at least 48 hr. in natural sea water, of which the chlorinity was determined concurrently with the analysis of the blood. This sea water was in all cases of very nearly the same concentration as that from which the crabs had been originally taken, and for such small differences as may have existed a period of 48 hr. is ample for complete re-equilibration of the blood.

TABLE 1. PREVIOUS ANALYSES OF THE BLOOD OF *CARCINUS*, THE CONCENTRATION OF EACH ION BEING EXPRESSED AS A PERCENTAGE OF THE CONCENTRATION IN WHICH IT OCCURRED IN THE WATER FROM WHICH THE CRABS WERE TAKEN

Author	Bialaszewicz	Bethe	Bethe and Berger
Locality	Naples	Naples	Heligoland
Chlorinity of sea water	22·27	22·1	19·20
Na	—	—	125
K	—	130	92
Ca	119	119	118
Mg	79	46	58
Cl	103	98	101
SO_4	89	—	—

The blood was withdrawn by inserting a glass cannula into the basal joint of one of the walking legs. It was stirred, centrifuged, and the plasma poured off the corpuscles, within five minutes of being drawn. The analytical methods used were those described by Robertson and Webb (1939), except that some of the sulphate estimations were performed by a barium iodate method (Webb 1939). Each sample analysed represents the combined blood of two, or sometimes three, individuals. In all cases the specific gravity and percentage of total solids were determined, so that the concentrations of the individual ions could be expressed in terms of weight of water instead of volume. They are expressed in table 2 as percentages of the concentrations in the external medium, the latter also being calculated in terms of weight of water. The protein figures, which must be regarded as only approximate, are obtained by subtracting the weight of salts from the weight of total solids.

It will be seen that the chloride content of the blood is almost identical with that of the sea water. The difference does not exceed 2 %, and of this about half may easily be due to experimental error. On the other hand the concentrations of sodium, potassium and calcium are considerably higher, and those of magnesium and sulphate much lower in the blood than in sea water.

TABLE 2. COMPOSITION OF THE BLOOD OF *CARCINUS* KEPT IN NORMAL SEA WATER. CONCENTRATIONS EXPRESSED, AS IN TABLE 1, AS PERCENTAGES OF THE CONCENTRATION IN THE EXTERNAL MEDIUM, BOTH CONCENTRATIONS BEING CALCULATED IN TERMS OF WEIGHT OF WATER

Sea water	Blood						
mg. Cl per g. of water	Protein (mg. per ml.)	Na	K	Ca	Mg	Cl	SO_4
18·71	63	108·7	123	125	38·3	97·7	62·2
18·64	59	114·4	—	—	—	98·5	—
19·73	56	115·5	—	—	43·2	101·9	—
18·67	72	112·7	119	136	36·0	98·4	65·0
19·78	66	100·8	135	118·5	28·0	101·0	51·6
19·83	43	113·9	109	130	33·1	102·0	50·2
Average	60	111·0	121	127	35·8	99·8	57·2

If, however, a true idea is to be obtained of the extent to which the composition of the blood differs from that which would result from a passive equilibrium, it is necessary, as has been emphasized by Robertson (1939), that a comparison should be made, not with the external medium, but with the blood after dialysis against this medium across a membrane permeable to water and to salts but not to proteins. This dialysed blood will differ in composition from the medium against which it is dialysed for two reasons: first, because of the redistribution of ions to fulfil the conditions of a Donnan equilibrium, and secondly because of the possible existence of undissociated compounds of some of the ions with proteins. Two samples of blood were accordingly dialysed against sea water across a relatively permeable collodion membrane. The outside solution (sea water) was stirred by a stream of air, and it was saturated with toluol to discourage bacterial action. The composition of the fluid thus obtained is compared in table 3 with the original blood and with the sea water against which it was dialysed. The high figure for calcium in the first column agrees with the findings of Robertson (1937) and indicates that some of the blood calcium is present in an indiffusible and undissociated form. The remaining ratios do not depart further from those expected in a Donnan equilibrium than may be accounted for by experimental error. Averaging the ratios for the univalent ions and the square roots of the ratios for the bivalent ions (other than calcium) a mean ratio of $r = 1{\cdot}026$ is obtained, where

$$r = \mathrm{Na}_i/\mathrm{Na}_o = \mathrm{Cl}_o/\mathrm{Cl}_i = \sqrt{\mathrm{SO}_{4_o}/\mathrm{SO}_{4_i}} \text{ etc.}$$

This is somewhat higher than the ratio found by Dailey *et al.* (1931) for the blood of *Limulus*, but in their case the protein content was only 2·5 %, whereas in the samples analysed in table 3 it was at least double this figure. Furthermore, all the protein in the blood of *Limulus* appears to be haemocyanin, which has a relatively low base-binding capacity, whereas in *Carcinus* a comparison of the copper content (average 70γ per ml.) with the protein content (average 5·5 g. per 100 ml.) shows that over half the protein must consist of albumins and globulins. For human serum, which contains about 7·5 % of proteins, mostly albumin and globulin, the Donnan ratio is about 1·04 (Van Slyke 1926), so that a figure between 1·025 and 1·03 seems reasonable for the blood of *Carcinus*.

TABLE 3. COMPOSITION OF BLOOD BEFORE AND AFTER DIALYSIS AGAINST SEA WATER

	Concentration in dialysed blood as % of concentration in sea water	Concentration in original blood as % of concentration in dialysed blood
Na	101·3	109·6
K	102·9	117·6
Ca	117·9	107·9
Mg	104·3	34·3
Cl	96·3	103·6
SO_4	94·2	60·7

It appears therefore that by some active process *Carcinus* is able to raise the concentration of potassium, sodium, calcium and chloride in its blood, and to lower that of magnesium and sulphate with respect to the equilibrium values. It is important to notice that the chloride content of the blood is greater than the equilibrium value, although it is usually less than that of the outside water, especially when expressed in volumetric terms and not with respect to the water content of the fluids. The observations made by Bateman (1933) on chloride concentration lead to the same conclusion. It is true that Duval (1925) found that the chloride concentration of the blood of Mediterranean specimens was only 90 % of that of the water, expressed in terms of volume. This, corrected for water content, corresponds to about 94·5 %, which, even allowing for the Donnan effect, is well below the equilibrium value. But there is reason to suspect some of

Duval's chloride analyses in so far as the ratio which he obtained for the freezing-point and chlorinity of sea water ($\Delta/\mathrm{Cl}_v = 0{\cdot}102$) differs by 6% from that derived from the formulae given by Thompson (1932), which are universally used in oceanography ($\Delta/\mathrm{Cl}_v = 0{\cdot}096$).

If the figures in table 3 are compared with similar figures given by Robertson (1939) for *Homarus* and *Cancer*, it will be seen that the general tendency is the same in all three genera. In *Homarus* the departure of the concentrations of potassium, magnesium and sulphate from the sea-water values is more marked than in *Carcinus*; in *Cancer* it is rather less marked. The figures for sodium are very much the same in all three. From this it may be inferred that no simple correlation exists between powers of osmotic and of ionic regulation, for with respect to ionic regulation *Carcinus* is intermediate between *Cancer* and *Homarus*, but with respect to osmotic regulation it is *Cancer* that is intermediate between the other two, since it possesses a limited power of osmoregulation (Duval 1925; Schlieper 1929*b*) which is completely lacking in *Homarus*. Nevertheless, as will appear later, the mechanisms underlying the two processes are very similar.

PREVIOUS EXPLANATIONS OF IONIC REGULATION

That there is a general tendency throughout the animal kingdom for the body fluids to be rich in potassium, and poor in magnesium and sulphate, in relation to the concentrations in the environment, is clearly brought out by the analyses of Bethe and Berger (1931), Bialaszewicz (1933) and Macallum (1926). As far as the accumulation of potassium is concerned, the phenomenon is also to be observed in the majority of animal and plant cells, and has been studied most intensively in mammalian erythrocytes and in certain algae such as *Valonia*. The erythrocyte appears to act as a passive, cation-impermeable system, in true equilibrium with the plasma, retaining in its interior for the course of its comparatively brief life the store of potassium with which it started. It is therefore of little relevance to the problem under discussion. In the case of the large plant cells the equilibrium must be an active one, but in other respects, for example the exclusion of sodium, the phenomena differ from those observed in the ionic regulation of the body fluids of animals, and it is by no means certain that the same interpretation will serve for both processes. Osterhout and his school, and also Brooks (1929) and Briggs (1930) have attempted explanations of the accumulation of potassium by plant cells of this type. All such schemes require the constant production within the cell of carbon dioxide or an organic acid. Such a state of affairs (the redistribution of ions about a membrane, on account of the production on one side of it of an electrolyte, of which the two constituent ions penetrate the membrane with different velocities) has been studied in a generalized form by Teorell (1935, 1937) and Meyer and Sievers (1936). Teorell has pointed out that the Donnan equilibrium is only a special case of this condition, in which the velocity of one of the ions through the membrane is zero. His figures would seem to render it very doubtful that any living system is capable of producing continuously an electrolyte of the requisite properties, in sufficient quantity to bring about any significant redistribution of the solutes in a solution as concentrated as sea water. In any case all work on *Valonia* needs reconsideration in view of the recent demonstration by Brooks (1938, 1939) that alternating phases of accumulation and release of cations by the protoplasmic layer must greatly complicate the kinetics of their penetration into the sap.

Of explanations dealing more specifically with the body fluids of marine invertebrates those of Scholles (1933) and Pantin (1931*a*) demand consideration. Scholles, from his own analyses and those of Bialaszewicz (1932), came to the conclusion that ionic regulation in Crustacea could be attributed entirely to the powers of differential excretion possessed by the antennary gland. As will be seen below, this is an important factor, but one which cannot by itself account for the difference between the composition of the blood and that of sea water. Pantin, writing before any analyses of the urine of marine invertebrates had been published, suggested that even if the urine were identical in composition with the blood, differences between the composition of the blood and that of the surrounding water could arise from differences in the rates at which the various ions penetrated the body surface. The flaw in this idea has been pointed out by Robertson (1939), who argues that a potassium ion, no matter how great its mobility, will never penetrate inwards through the body wall against a concentration gradient by virtue of diffusion alone. The weak point in Pantin's analogy of a leaking vessel being continuously baled out is that there is in the animal nothing to correspond to the hydrostatic pressure which causes the water to enter the boat, for no animal maintains a negative blood pressure. And even if, through vital activity of the membrane, water were enabled to enter at the appropriate rate, it is clear, for the reasons given by Robertson, that the system could apply only to an animal whose blood had lower concentrations of *all* the ions in question than those in the external medium, and was therefore markedly hypotonic. This state of affairs may well exist in such genera as *Pachygrapsus* (Baumberger and Olmsted, 1928), *Heloecius* (Dakin and Edmonds, 1931), *Leptograptus*

(Edmonds, 1935), *Eriocheir* (Conklin and Krogh, 1938), *Palaemonetes* (Panikkar, 1939), and *Leander* (Panikkar, 1940). All these forms, when living in sea water, maintain their blood hypotonic to the environment. Analyses for single ions have been made only on the blood of *Eriocheir*, and these are incomplete and probably not very accurate; but it is quite possible that in some instances the concentration of all ions is lower in the blood than in the environment. If this were true, the steady state considered by Pantin would be realized, and might become the determining factor in ionic regulation, but it certainly cannot arise in such forms as *Carcinus*, which, as Pantin himself points out, is if anything somewhat hypertonic to its environment.

THE PERMEABILITY OF THE BODY SURFACE

(1) *Evidence for permeability to water and salts.* For many years all discussions on the osmotic relations of aquatic animals were conducted on the assumption that their outer surfaces behaved virtually as ideal semi-permeable membranes. This view, although it had been questioned before, was first challenged effectively by Bethe (1929) who claimed to show that the surfaces of *Aplysia* and *Carcinus* were permeable to salts as well as to water. The experiments which he described in this paper have been subjected to a certain amount of criticism, but Bethe's general thesis must now be taken as proved beyond all reasonable doubt (see Berger 1931; Berger and Bethe 1931; Bethe 1934). The only recent dissentients from this view have been Adolph (1936) and Maloeuf (1937). The former measured the changes of weight in *Phascolosoma* when immersed in hypotonic and hypertonic media, and claimed that the animal acted as a semi-permeable osmometer, but, as Krogh (1939) points out, his data do not really support this contention. Maloeuf found that the isolated gills of *Cambarus* could be made to swell and collapse repeatedly by immersion alternately in distilled water and a salt solution hypertonic to the blood; this was held to show that the gills were semi-permeable even after the cells might be presumed to have been killed. Probably his experiments were of too short duration to be regarded as conclusive, and his observation in the same paper, that when a crayfish dies by exposure to too strong a salt solution its blood is found to have become isotonic with the external medium, is scarcely compatible with an impermeability of the integument to salts.

It may therefore be taken as established that in general the surface of aquatic animals is to some extent permeable to salts. Specific observations on *Carcinus* are not very numerous, but some of them seem to be fairly conclusive. Nagel's experiment mentioned below (p. 127) gave a clear proof that under certain conditions chlorides can pass inwards through the body surface. Some of Bethe's (1929) original experiments leave little doubt that magnesium, the least mobile of the cations in sea water, can diffuse inwards, and Berger and Bethe (1931) showed that not only the body surface as a whole, but even the calcified cuticle of the carapace, possesses a measurable permeability for iodides.

No sooner was it generally agreed that the integument of *Carcinus* is permeable to salts than it was suggested that it is impermeable to water. This idea seems to have originated in an *obiter dictum* by Hill (1931), and was given wider currency by Bateman (1933). It is difficult to see how it can ever have been taken seriously. Already Margaria (1931) had given a generally accepted explanation of the dilution curve of the blood in terms of permeability of the surface to both water and salts. It is well known that excretion of water through the antennary gland goes on continuously even in full-strength sea water, and that this water must enter through the integument, since Nagel (1934) and others have shown that blocking up the mouth makes no difference to the salt and water exchange. Finally, a further proof is provided by the experiments of Bethe, von Holst and Huf (1935) and Huf (1936), who showed that *Carcinus* gains rapidly in weight in diluted sea water if the regulatory mechanism is interfered with by blocking up the antennary glands.

Many of the observations reported in this paper are quite inexplicable unless permeability of the surface to water and salts is conceded, but the point need not be laboured further.

(2) *The permeability of the cuticle.** The only systematic investigation that has been made into the permeability of the crustacean cuticle is that of Yonge (1936), who used the uncalcified and relatively permeable lining of the foregut of *Homarus*. Some of his conclusions are rather startling. The permeability of the cuticle as a whole is effectively controlled by that of the epicuticle, the chitinous layer being freely and rapidly permeable to water and salts. Chlorides penetrate the intact cuticle in accordance with the mobility of their cations (except for NH_4Cl, which is abnormally fast), but the bivalent cations ($Ca^{\cdot\cdot}$, $Mg^{\cdot\cdot}$, $Ba^{\cdot\cdot}$, $Cd^{\cdot\cdot}$) are slowed up enormously in comparison with the univalent. The most remarkable finding is that in all cases these bivalent cations penetrated more rapidly in one direction

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* By *cuticle* is meant the whole of the non-cellular membrane that covers a crustacean. The outer, partly fatty, layer is called the *epicuticle*, the remainder the *chitinous layer*. By *integument* is denoted the cuticle together with the hypodermis. This nomenclature differs from that of Yonge (1932, 1936), but has the advantage of conforming to that used for other arthropods.

(chitin to epicuticle) than in the other. Ammonia and fatty acids, on the other hand, penetrated only in the reverse direction (epicuticle to chitin) in experiments in which the membranes were suspended in air with the solution in contact with their upper side. These latter conditions are not of much physiological interest, but they led Yonge on to perform a series of experiments with ammonia and fatty acids, in which there was initially the same concentration on either side of the membrane. He claimed, though with some diffidence, that in all cases an accumulation of the solute took place on the side next the chitin, the concentration differences observed being of the order of 4 %.

If the crustacean cuticle really possesses these remarkable properties of differential and irreciprocal permeability it is clear that it might, without any assistance from the underlying epithelium, produce a considerable departure in the composition of the blood from the expected equilibrium condition. I have accordingly repeated a number of Yonge's experiments. A full account of these investigations, which are not yet completed, will be published elsewhere; in the meantime it is possible to summarize those results which bear on the behaviour of the cuticle under natural conditions, and to offer some criticisms of Yonge's procedure, which, though they do not attempt to set aside his results, may give a partial explanation of their peculiar nature.

In general it may be said that my experiments show that *under conditions which are approximately physiological* the crustacean cuticle gives no evidence of irreciprocal permeability, and that the relative velocities with which it is penetrated by the principal ions found in sea water are similar to those with which they penetrate cell membranes. By "approximately physiological conditions" is meant that the solutions on either side of the membrane shall contain only chlorides and sulphates of the alkalies and alkaline earths, that they shall be balanced with respect to the concentrations in which different cations are present, and that their total concentration shall be of the same order of magnitude as that of sea water (say between 0·12 and 1·2 M).

One of the disadvantages of the apparatus employed by Yonge was its assymmetry: that is to say the diffusion path was freer on one side of the membrane than on the other, a point which is all the more important since neither solution was stirred. This cannot in itself account for Yonge's anomalous results, but it greatly complicates their interpretation. A more important point concerns the recent history of the membranes, and their changes in permeability in accordance with the nature of the solution that bathes them. Yonge worked throughout with pure solutions of a single substance. But as there is reason to suppose that the epicuticle contains proteins as well as lipoids (Yonge 1932), it is *a priori* probable that its properties will be affected by the ionic balance of the surrounding medium. In practice it is easy to show that the addition of calcium chloride, sufficient to give a concentration of 0·01 M, to a 0·6 M solution of sodium chloride decreases the permeability of the membrane to sodium by about 50%. This is in accordance with the well-known property of calcium of maintaining impermeability (cf. Pantin 1931 *b*). Under the conditions used by Yonge the difference in penetration rates between sodium and calcium chlorides is due partly, no doubt, to the real difference in permeability of the membrane for $Na^{\cdot}$ and $Ca^{\cdot\cdot}$ ions, but much more to the greatly diminished permeability *to all ions* induced by the presence of calcium. It is also important to note that a membrane that has been immersed in distilled water or pure sodium chloride solution is much more permeable than one that has been kept in sea water, and that it is a matter of some hours before these differences become adjusted in accordance with a new medium. It is quite conceivable that the reaction of the membrane to such changes might take place more rapidly for a change on one side of it than for a change on the other, and that a certain degree of "irreciprocal permeability" might be brought about in this way.

Appropriate precautions were taken to eliminate all these complications. The apparatus used was symmetrical with respect to the plane of the membrane, and the solutions on both sides were kept in motion. The solutions used were always concentrated or diluted sea water, and before each experiment the membrane was soaked for several hours in a mixture of equal parts of the two solutions to be used.

Under these conditions, as has been stated above, no evidence of irreciprocal permeability was found. The relative velocities of permeation of the different ions are set out in table 4. It is impossible, owing to the complexity and interaction of the electrical and concentration gradients set up across the membrane in such a system as this, to calculate absolute permeability constants for the various ions, and accordingly $Na^{\cdot}$ and Cl' have been taken as standards and given arbitrary velocities of 100. The figures agree remarkably closely with those given by Koizumi (1935 *a*, 1935 *b*) for the relative permeabilities for the same ions of the isolated integument of the holothurian *Caudina*, which may, for this purpose, be presumed to be typical of inactive and unspecialized epithelia. It would appear, therefore, that as far as the penetration of salts is concerned the presence of the cuticle will not produce any effect which is not already produced by the presence of the underlying epithelium. At most it may to some small extent exaggerate the difference between the velocities of the faster and slower ions.

TABLE 4. RELATIVE PERMEABILITY OF THE CUTICLE OF THE FOREGUT OF *HOMARUS* TO DIFFERENT IONS

Na	100	Cl	100
K	169	SO_4	52
Ca	72		
Mg	42		

The absolute permeability to water of the material studied (lining of the foregut of *Homarus* and *Cancer*) varies considerably from one sample to another, but is usually of the order of $0{\cdot}4\mu^3/\mu^2$/min./atm. If this figure is compared with the available data for the permeability of the surface of various cells (references will be found in Krogh (1939) and Lucké, Hartline and Ricca (1939)), it will be seen that the crustacean cuticle is rather more permeable to water than the majority of cell membranes, though the figures are of the same order of magnitude. It must be remembered, however, that the epicuticle of this material is about a hundred times as thick as that which covers the gills (Yonge 1932), and although there is as yet no evidence to show how much of the resistance to the penetration of water resides in the substance of the epicuticle and how much at its surface, it seems reasonable to presume that the cuticle of the gills is at least three or four times as permeable as the cuticle of the foregut. Since, as is shown in the next section, most of the water exchange takes place through the gills, it follows that the epithelium is far more likely than the cuticle to be the factor which effectively controls the water flow.

It may therefore be concluded that, whatever the properties exhibited *in vitro* by the crustacean cuticle may be, it is unlikely under normal conditions to have much influence on the salt and water exchange between the animal and its environment. Further evidence in support of this conclusion may be derived from a study of the structure of the gills.

THE GILLS

Bethe (1929), in his original observations on the permeability of the body surface of marine invertebrates to ions, observed that *Aplysia* responded, by changes both in its behaviour and in the composition of its blood, to alterations in the composition of the external medium far more rapidly than did *Carcinus*. He argued from this that diffusion of salts must take place through the body wall in general, rather than through the gills, since *Carcinus* has a far greater gill area than *Aplysia*, but a general body surface that is obviously much less permeable. This conclusion is open to objection on two grounds. It assumes, in the first place, that the permeability of the gills of two unrelated animals is likely to be about the same per unit area, for which there is no evidence. In the second place it fails to take into account the regulatory function of the antennary gland of *Carcinus*, which tends, by varying the rate of excretion of any ion in accordance with the rate at which it is entering the body, to prevent, or at any rate to slow up, changes in the composition of the blood. In point of fact it may be deduced from later experiments on iodide absorption (Berger and Bethe 1931) that in *Astacus* practically all the exchange of salts must take place through the gills, the rest of the body surface being responsible for 3 or 4 % at the most, and perhaps much less. These authors conclude that this is to be explained rather by the large area of the gills than by their especially high permeability, and quote in support of this view an estimate by Pütter (1911) of 60 sq. cm. as the gill area of a crayfish weighing 20 g. Estimates which I have made on *Carcinus*, by counting the gill-lamellae and measuring, in a representative sample, the area of each, yield a rather lower figure, namely 50 sq. cm. for a body weight of 80 g. This difference is possibly correlated with the fact that *Carcinus* is phyllobranchiate and *Astacus* trichobranchiate. But even if we take the figure for *Astacus* the area of the gills is not much greater than that of the rest of the body surface, and it follows that they must be considerably more permeable.

Granted then that the gills are the main path of salt and water exchange, it is of interest to examine their structure in species which possess in various degrees the power of osmotic or ionic regulation. Little has been published on the histology of the decapod gill; there is a general survey by Bernecker (1909) and special studies by Bock (1925) on *Astacus*, Chen (1933) on *Grapsus* and Allen (1892) on *Palaemonetes*, but much of this work, particularly of the last two authors cited, is concerned with structures irrelevant to the present problem. Sections were accordingly prepared of the gills of four representative marine species: *Maia squinado*, which is stenohaline, poikilosmotic, and with only rather feeble powers of ionic regulation (Bialaszewicz 1932); *Carcinus maenas*, which is euryhaline, partly homoiosomotic, and with considerable powers of ionic regulation; *Homarus vulgaris*, with even more highly developed ionic regulation (Robertson 1939), but stenohaline and poikilosmotic; and *Eriocheir sinensis*,* euryhaline and homoiosmotic to a greater extent even than

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* The *Eriocheir* material was obtained through the courtesy of Professor W. Arndt, of the Berlin Museum.

Carcinus, and possessing also considerable powers of ionic regulation (Scholles 1933). The structure of the gills of these four species is illustrated in figures 1–4, plate 7.

Since the euryhaline forms, *Carcinus* and *Eriocheir*, not only maintain an osmotic difference between their blood and the external medium, but also respond far more slowly than stenohaline forms to variations in the concentration or composition of the medium, it has been claimed that their surface is much less permeable to water and salts than that of the stenohaline forms such as *Maia* (Schwabe 1933; Nagel 1934; Conklin and Krogh 1938; Krogh 1939). An examination of the photographs in plate 7 shows that if this is true it must certainly be ascribed to differences in the epithelium and not in the cuticle. The cuticle of the gill lamellae is, perhaps, slightly thicker in *Carcinus* than in *Maia*, but in *Eriocheir*, which of all Crustacea hitherto examined is the most "water-tight" it is apparently absent altogether. This remarkable fact cannot be ascribed to a fault in technique or to an abnormal condition of the individual examined, for the cuticle is well developed and quite plainly visible near the margins of each gill lamella. On following it towards the centre it rapidly becomes thinner and soon, without any sudden break, ceases to be perceptible, except in very oblique sections, where it can sometimes be seen as a faint ghost-like film, of which the thickness is clearly below the limit of resolution of the microscope. At least 80% of the gill surface is virtually naked. In the three other species the thickness of the cuticle is fairly uniform over the whole surface of the lamella. The epicuticle is about $0 \cdot 5\mu$ thick, although in the photographs it appears much thicker; this is owing to the fact that it inevitably lies somewhat obliquely on the slide in a section which is 5μ thick. The chitinous layer is considerably thicker in *Homarus* than in *Maia* or *Carcinus*, but this is presumably without significance as far as permeability is concerned. Bock's (1925) figures show that the same thick chitinous layer is found in *Astacus*.

These sections, therefore, provide further evidence for supposing that the cuticle does not control the permeability of the gills or the animal's power of regulation, and that the epithelia must be responsible for the phenomena which have lent support to the idea of relative impermeability of the gills in *Carcinus* and *Eriocheir*, and also in *Astacus* (Huf 1933). But if the epithelium is responsible it is possible to explain the facts equally well in terms of active salt absorption as by assuming a passive impermeability. Reasons for preferring the former explanation are given later, but in the meantime attention may be drawn to the differences shown in plate 7 between the branchial epithelia of the different species. The thickness of the cytoplasmic layer interposed between the cuticle and the outermost blood lacunae cannot be of any significance for the permeability of the epithelium, since the obstacle to the diffusion of water and ions lies not in the substance of the cytoplasm but in its bounding membranes. But the general development of the branchial epithelium may reasonably be presumed to afford some criterion of the extent to which active processes such as salt absorption are being carried out by it. It is quite clear that the epithelium of *Maia* is feebly developed in comparison with that of the other forms; it is also, of the four species. that with the feeblest regulatory powers. Comparison of the trichobranchiate *Homarus* with the phyllobranchiate species is rather difficult, since the whole gill structure is rather different, but it would appear, judging by the figures of Bock (1925), that among the trichobranchiate forms the epithelium of *Astacus* (homoiosmotic) is better developed than that of *Homarus*.

These conclusions drawn from the differences in the branchial epithelium are not quite so conclusive as those afforded by a study of the cuticle, but they give a certain amount of support to the theory, which is developed below, that salt absorption by the gills is a *sine qua non* of osmotic and ionic regulation.

THE ROLE OF THE ANTENNARY GLAND

Attention has several times been drawn to the presence in the antennary gland of freshwater Malacostraca of a long segment, the nephridial canal, interposed between the labyrinth and the urinary bladder. In marine species, often of the same genus or family, this segment is short or absent. (For examples and a few exceptions see Schwabe (1933) and Krogh (1939).) The first definite function, from the point of view of salt and water balance, to be ascribed to the antennary gland was the absorption of salts by this nephridial canal, so that species living in a hypotonic medium should be enabled to excrete the large quantities of water which must be presumed to enter osmotically, without at the same time losing salts, which for these species are valuable and hard to replace. That this in fact takes place in the crayfishes *Astacus* and *Cambarus* has been shown by Herrmann (1931), Scholles (1933), Peters (1935), Picken (1936) and Lienemann (1938). It seemed probable therefore that these conclusions could be extended to other homoiosmotic forms, and that osmotic regulation in general could be ascribed to a varying degree of salt conservation by the antennary gland. But the observations of Schlieper (1929 *a*) on *Carcinus*, Schlieper and Herrmann (1930) on *Telphusa*, Scholles (1933) on *Eriocheir*, and Nagel

(1934) on *Carcinus* showed that these species, even when living in water hypotonic to their blood, excreted a urine isotonic with their blood. It would appear therefore that the antennary gland in these euryhaline forms can have no osmoregulatory function.

It was from a knowledge of the earlier of these observations that Pantin's hypothesis, discussed on p. 113, was put forward. The criticisms to which this particular hypothesis is open do not mean that Pantin's general point may not be true—that the antennary glands may bring about ionic regulation even if the urine is isotonic. At the time that he wrote no analyses of the urine of Crustacea were available. Since then figures have been published by Bialaszewicz (1932), Scholles (1933) and Robertson (1939) for marine forms, and by Scholles (1933), Bogucki (1934) and Lienemann (1938) for freshwater crayfish. The latter are of less interest for our problem, since in freshwater species there is no constant relation between the concentrations of the different ions in the blood and in the external medium. The data for the marine forms are drawn up in table 5. It is apparent that something more than ultrafiltration is being carried out by the antennary gland, and that the differential retention and excretion of ions which may be deduced from these figures may well be an important factor in ionic regulation. The composition of the urine of *Cancer* and of *Maia* is very similar, and is very close, especially in *Maia*, to that of sea water. In both cases potassium is retained, magnesium and sulphate selectively excreted, and sodium and chloride passively excreted, their concentration in blood and urine being much the same. This is what might be expected from the high potassium and low magnesium and sulphate concentrations in the blood. The case of *Eriocheir* is rather different, since it maintains a hypotonic blood when living in sea water.

TABLE 5. COMPOSITION OF THE URINE OF MARINE CRUSTACEA, FROM THE DATA OF PREVIOUS AUTHORS. CONCENTRATIONS OF EACH ION ARE EXPRESSED AS PERCENTAGES OF THEIR CONCENTRATIONS IN THE BLOOD

Species ...	*Maia squinado*	*Eriocheir sinensis*		*Cancer pagurus*
Author ...	Bialaszewicz (1932)	Scholles (1933)		Robertson (1939)
		Sea water	Fresh water	
Na	—	—	—	97
K	82	115	139	80
Ca	122	93	47	89
Mg	139	222	22	125
Cl	102	113	95	96
SO_4	145	—	—	133

As no data have hitherto been published on the composition of the urine of *Carcinus*, several analyses were made of samples collected from crabs living in both normal and modified sea water. The urine was collected by raising the operculum at the opening of the gland and receiving the fluid in a cannula as it ran out. If the cannula is inserted into the bladder, there is always a risk of laceration of the wall and contamination of the urine with blood.

Table 6 represents the average of five separate analyses of the urine of crabs which had been kept for 48 hr. in normal sea water containing 19·20 g. Cl per litre. It will be seen that, as in *Maia* and *Cancer*, there is retention of potassium and elimination of magnesium and sulphate, but that with regard to the last two ions the selective excretion is far more pronounced in *Carcinus* than in the other forms. The concentrations of magnesium and sulphate in the urine considerably exceed not only those in the blood but also those in the external medium.

TABLE 6. COMPOSITION OF THE URINE OF *CARCINUS* KEPT IN NORMAL SEA WATER

	mg. per g. water	As % of average concentration in the blood
Na	11·14	95
K	0·352	78
Ca	0·475	94
Mg	1·805	390
Cl	18·80	98
SO_4	3·42	224

As a further test of the importance of this function of the antennary gland, urine was collected from crabs that had been kept for 36 hr. in a mixture of sea water (25 parts) with isotonic (0·81 M) magnesium sulphate (1 part). By this means the magnesium content of the external medium is increased by 56 % and the sulphate content by 108 %. Under these conditions, as may be seen from table 7, which shows the concentration of certain ions in the blood and urine, an increased activity of the antennary gland in eliminating magnesium and sulphate keeps the concentration of these ions in the blood down to a level very little above the normal.

To obtain a complete idea of the importance of the antennary gland in ionic regulation it is necessary to know not only the composition of the urine, but also the rate at which it is produced. Estimates of this rate have been made for several species of decapods, including *Carcinus*, by previous workers, and are summarized in table 8. The variation in the urinary output of *Carcinus* in accordance with the concentration of the external medium need not concern us at the moment, but it will be seen that there is a discrepancy between Nagel's figures and those of Bethe *et al.*, the former being about double the latter and considerably higher than the figures obtained for any other species. A few estimates which I have made of the rate of urine production in full-strength sea water gave an average value of 4·7 % of the body weight per day. This is in reasonable agreement with the figure of Bethe *et al.* (taking into account the difference in concentration of the external medium), and I am inclined to think that Nagel's figures are too high. The method used was the same as that used by both these authors—weighing over intervals not exceeding 12 hr. crabs whose antennary gland openings had been sealed with dental cement. For the purposes of the subsequent discussion it will be assumed that the urinary output of *Carcinus* in normal sea water is 5 % of the body weight per day.

TABLE 7. COMPOSITION OF THE BLOOD AND URINE OF *CARCINUS* KEPT IN A MEDIUM CONTAINING EXCESS MAGNESIUM AND SULPHATE

	External medium	Blood		Urine	
	% of concentration in normal sea water	% of concentration in external medium	% of concentration in normal blood	% of concentration in blood	% of concentration in normal urine
Na	96	112·5	99	92	96
Mg	156	24	104	500	133
Cl	96	99	95	98	95
SO_4	208	29	108	465	223

TABLE 8. RATE OF URINE FLOW IN DECAPOD CRUSTACEA

Genus	Author	Urine flow, as % of body weight per 24 hr.
Maia	Bialaszewicz (1932)	3
Cancer	Robertson (1939)	3–10
Eriocheir (in fresh water)	Scholles (1933)	4·2
Carcinus (in sea water)	Nagel (1934)	10
Carcinus (in 50 % sea water)	Nagel (1934)	17
Carcinus (in 67 % sea water)	Bethe *et al.* (1935)	7·2
Potamobius	Herrmann (1931)	3·8
Cambarus	Lienemann (1938)	5·2

Little meaning can be attached to these figures until their relation to the blood volume is known. This has been estimated by Nagel (1934) as 37 %

of the body weight in *Carcinus*, and by Krogh (1938 *c*) as 33 % in *Eriocheir*. Both these authors relied on injection methods, the former using iodide, the latter thiocyanate. Three determinations which I have made on *Carcinus*, by estimating the total quantity of chloride in the body, and assuming that it is all in the blood, yield a very similar figure, namely 36 %. The necessary assumption that the intracellular chloride amounts to less than 5 % of the total is probably justified, since although Bear and Schmitt (1939) and Webb and Young (1940) have shown that chlorides are present in considerable quantities in the nerve fibres of cephalopods, the analyses of Manery (1939) and of Krogh (1938 *b*) suggest that in muscle and other tissues which contribute largely to the total weight of the animal the concentration of intracellular chloride, even in marine invertebrates, is so low that it is unlikely to cause more than a very small error in estimating the blood volume by this means. 35 % may therefore be accepted as a round figure for the blood volume of *Carcinus*, and probably also of other crabs.

This figure may at first sight seem rather startling in comparison with that for the blood volume of mammals (*c*. 7½ % of the body weight), but it must be remembered that the comparison should be made, not with the blood, but with the total volume of extracellular fluid, since in the Crustacea the blood fulfils also the functions of lymph and other transudates in mammals. Since the extracellular fluid of mammals amounts to 25–30 % of the body weight (Harrison, Darrow and Yannet 1936), a figure of 35 % for *Carcinus* is not unreasonable.

It may therefore be concluded that the antennary gland of *Carcinus*, when it is living in sea water, has a daily output equivalent to 14 % of the blood volume. This implies an activity considerably greater than that of the human kidney, which secretes daily a volume of urine equal to 25 % of the blood volume, but to only 7½ % of the volume of the extracellular fluid. Since it has already been shown that this excretion is characterized by a very considerable power of differentiating between the various ions present in the blood, it must be concluded that the antennary gland is a very important part of the mechanism of ionic regulation. This may be illustrated by two calculations. If it is assumed that the magnesium and sulphate enter the body by diffusion through the gills, an assumption which will be justified later, and that the rate of entry is therefore proportional to the difference in concentration between blood and external medium, it may be shown that a reduction by 50 % in the rate of secretion by the antennary gland, the composition of the urine remaining the same, would raise the blood magnesium by 72 % and the blood sulphate by 35 %. If on the other hand the volume of urine secreted remained the same, but its composition were that of a blood ultrafiltrate, the blood magnesium would rise by 92 % and the blood sulphate by 32 %. Similarly the concentration of potassium in the blood would fall, though the effect in this case would not be so pronounced.

THE REGULATORY MECHANISM

(1) *The absorption of salts.* Our whole picture of the salt balance of fresh-water animals has been radically changed within the last three years by the very important researches of Krogh (1937 *a*, 1937 *b*, 1938 *a*, 1939) on the active absorption of ions by these animals from very dilute solutions. Previously it seems to have been believed that the only source from which a fresh-water animal could replenish the salts lost in the urine and by diffusion through the body surface was its food. But numerous species can live for many weeks without taking food, and without allowing the salt content of their blood to fall by more than a few per cent. The assumption made by many authors (e.g. Scholles 1933) that in these circumstances the blood draws on stores of salts laid up in the tissue cells is demonstrably false; for although Bethe (1929) showed that in *Carcinus* considerable quantities of calcium could be mobilized from the cuticle, and of potassium from the tissues, when the supply of these ions in the external medium was cut down, his conclusions cannot be applied to all ions alike. It is universally acknowledged that those which are likely to be lost in the largest quantities by excretion and diffusion, namely sodium and chloride, are either absent altogether from the tissue cells, or else present in amounts so small as to be useless as a store. These considerations apply also to the urinary excretion of salts by starving individuals of poikilosmotic, iosotonic forms such as *Maia*. Even Krogh (1939) in this instance assumes that the tissues are the source of the salts, but a comparison of the figures given by Bialaszewicz (1932) for urinary output and composition, with those of Krogh himself (1938 *b*) for the composition of crustacean tissues, shows that, even at the most favourable estimate, the stores would last for only 3 or 4 days, whereas in fact the animal can survive starvation for several weeks.

The work of Krogh, and also of Koch (1938) and Wigglesworth (1938), has, however, shown that a very large number of freshwater animals can absorb certain ions (especially sodium and chloride, usually potassium as well, and sometimes calcium) through their integument against a concentration gradient. The experiments have for the most part been preceded by a "washing out" of the animal in distilled water, that is by exposing it to conditions in which salt loss is inevitable, till the concentration of its body fluids becomes subnormal. It is then transferred to tap water, natural stream water, 1/100 Ringer, or some similar solution in which salts are present, but in concentrations of the order of 1 % of those in the body fluids. The absorption of salts from such solutions has been demonstrated in frogs, fish, molluscs, crustaceans, insect larvae and leeches. The absorption must be presumed to go on continuously under normal conditions, and the preliminary washing out, which is necessary to demonstrate the process, is not necessary to initiate it. Normally the active absorption of salts exactly balances the loss that takes place by diffusion and excretion.

It would seem that the phenomena both of osmotic and of ionic regulation are best explained by supposing that this process of active salt absorption goes on not only in dilute media, but also in full-strength sea water, though it is carried out more vigorously in the former. It has already been pointed out that the concentrations of sodium, potassium, calcium and chloride in the blood of crabs which are living in sea water cannot be explained by diffusion and excretion alone, since they are higher than the equilibrium values. It is significant that these four ions are those which Krogh finds to be actively absorbed by fresh-water animals. Magnesium and sulphate are not actively absorbed, so that their concentration in the blood of *Carcinus* is the resultant of the processes of inward diffusion and differential excretion. The analyses set out in table 7 support this idea, as they show that the urinary output of magnesium and sulphate, which is of course equal to the quantity which enters through the body surface, is proportional to the concentration difference between the external medium and the blood.

That active absorption of salts can, in certain circumstances, take place in *Carcinus* is strongly suggested by the results of an experiment made by Nagel (1934). He demonstrated directly the fact that on transferring crabs from one dilution of sea water to another, slightly more concentrated, but still more dilute than the blood, there is a rise in the chloride content and osmotic pressure of the blood. As this is not accompanied by any fall in weight, salts must have been absorbed against a concentration gradient.

This power of active salt absorption must also be attributed to stenohaline forms such as *Maia*, even though they never make use of it for osmotic regulation. It has been shown that the salt loss in the urine of starving individuals cannot be made good from stores in the tissues: the salts must therefore enter through the body wall. Since the blood is isotonic with the external medium, this implies active transport. Here again magnesium and sulphate, being found in smaller concentration in the blood than in the sea water, enter by diffusion, while the other ions are actively absorbed. This theory explains, incidentally, why in all animals, and especially Crustacea, in which there is a continuous excretion of salts, there is some degree of ionic regulation, the magnesium and sulphate content of the body fluid being lower than that of the medium.

With regard to the maintenance of a potassium concentration in the blood higher than that in the medium, it seems justifiable, for *Carcinus* at any rate, to assume that the mechanism of active absorption discriminates little, or not at all, between sodium and potassium, so that the amount of these ions absorbed is in proportion to their concentration in the medium. There is a certain amount of passive diffusion of potassium in the opposite direction, but this is compensated for by the selective activity of the antennary gland, which excretes proportionately less of potassium than of any other ion. The factors influencing the calcium balance are more complex, as the stores in the cuticle tend to mask any temporary change in it; furthermore the indiffusible nature of much of the blood calcium must not be forgotten. On the whole it would appear from my analyses and those of Robertson (1937) that in the inter-moult phase a comparatively small amount of active absorption goes on, but this probably increases greatly after moulting.

A possible objection to the theory outlined above is Krogh's (1938 *a*) claim that sulphate ions do not diffuse into the blood of *Eriocheir* from the surrounding medium. This conclusion is, however, reached only by ignoring the activity of the antennary gland. The figures in table 7 for the blood of *Carcinus* living in sea water with excess magnesium sulphate might be cited as showing that, since in these conditions the sulphate concentration in the blood scarcely rises above normal, the integument is impermeable to sulphates. Actually the analysis of the urine shows that this is not so. In Krogh's experiments the sulphate was being returned to the medium in the urine as fast as it diffused through the integument, and hence its loss from the medium by diffusion into the blood could not be detected

(2) *The transport of water.* A study of the movement of water through the integument belongs rather to the province of osmotic than of ionic regulation, but it must be considered if the relation between the two processes is to be made out. The question to be decided is whether under normal conditions, that is to say when living in full-strength sea water, the water which enters the blood of *Carcinus* from the external medium, to compensate for the water lost in the urine, moves under the influence of

osmosis, or whether it is necessary to postulate an "active" absorption of water as well as of salts.

The principal point at issue is whether under normal conditions *Carcinus* is exactly isotonic, or whether there is always a small osmotic gradient tending to draw water into the blood. Both Schlieper (1929 *a*) and Schwabe (1933) claimed that *Carcinus*, except immediately after moulting, is hypertonic, the difference in freezing-points of blood and water being 0·05° (corresponding to about 2·5 % of the total osmotic pressure). On the other hand, Picken (1936) found some individuals hypotonic and others hypertonic, but the mean concentrations of blood and sea water showed no significant difference. I have made no direct observations on this point, but the analyses of normal blood in table 2 have some relevance. By calculating the total number of ion-equivalents of all types in both blood and sea water it may be shown that, assuming equal degrees of dissociation in both solutions, the osmotic pressure of the blood due to salts is about 0·7 % higher than that of sea water. To this must be added the osmotic pressure of the proteins and of diffusible organic solutes, but it is unlikely that these account together for more than 0·1 % of the total. It would seem therefore that even if the blood is, on the average, hypertonic to sea water, the difference is less than 1 %, and is probably not constant. Using the figures for gill area and urinary output which have been given earlier, it would appear that the movement of water inwards in sufficient quantity under an osmotic gradient equivalent to 1 % of sea water implies that the permeability of the gills is about $2\mu^3/\mu^2$/min./atm. This figure is far higher than that of all other epithelia and even of most single cell-membranes. It would seem probable, therefore, that other forces besides osmosis are at work.

There are other lines of evidence leading to the same conclusion. It has already been shown that the ionic regulation of *Maia* differs only in degree from that of *Carcinus*. Schwabe (1933) finds, however, that the blood of *Maia*, as of all stenohaline species, is exactly isotonic with the external medium, yet its urinary output is only slightly less than that of *Carcinus*. There is no option but to conclude that the uptake of water in this case is an active process, and there is no reason why the same should not be true for *Carcinus*.

Again, it is well known that Crustacea take in very large quantities of water immediately after moulting. Krogh (1939) believes that this water enters osmotically, as a result of a sharp rise in the concentration of the blood before the moult, and quotes figures from Baumberger and Olmsted (1928) in support of this. There is no doubt that these authors did observe a surprisingly large rise in the osmotic pressure of the blood immediately before the moult, but their deduction that water enters unaccompanied by salts has been shown by Drach (1939) to be based on an error in calculation. In any case it must be remembered that *Pachygrapsus*, the form on which they worked, is normally hypotonic to its environment, so that its osmotic relations are different from those of isotonic species. Krogh also maintains that the work of Robertson (1937) on *Carcinus* leads to the same conclusion. In point of fact Robertson showed no increase in the osmotic pressure of the blood before the moult, but merely a decrease in the water content of the whole crab, which is by no means the same. Furthermore, he showed that the water which enters the body after the moult is accompanied by salts in about the same concentration as in sea water. This has been since confirmed by Drach. It is therefore quite impossible that the water should have entered solely as the result of osmotic forces. The physiological condition of a crab during its moult is admittedly peculiar, but it is clear that in some circumstances at any rate an active transport of water through the integument can take place.

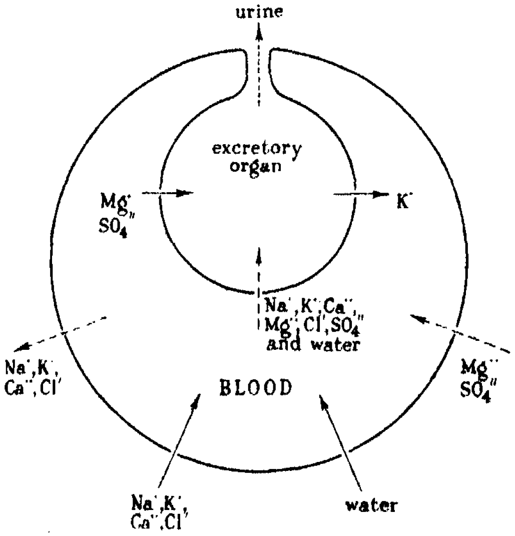

FIGURE 6. Diagram to illustrate the salt and water exchange of *Carcinus* when living in full-strength sea water. The broken arrows represent the movement of solutes or water under the action of "physical" forces (diffusion and filtration), and the unbroken ones their movement under "vital" forces, of which the physical basis is not known.

It might perhaps be suggested that the uptake of water is an inevitable concomitant of the absorption of salts. Until more is known about the nature of the latter process this must remain a matter for speculation, but it may be recalled that Schlieper (1933) claims to have shown that the excretion of salts through the gills of the eel against a concentration gradient is not accompanied by the excretion of any appreciable amount of water. A certain amount of water may be presumed to enter with the salts as water of hydration of the ions, but judging from the figures given by Bethe (1934) and Koizumi (1938) this could not account for more than about an eighth of the whole.

(3) *The relation between ionic and osmotic regulation.* The active absorption of salts forms the basis of the osmoregulatory mechanism, not only in freshwater forms, but also in certain brackish-water forms, including the euryhaline Crustacea. Since, according to the theory which has been outlined above, this process of absorption is also an essential of ionic regulation, it is clear that the two processes must be closely linked together. This connexion was emphasized on other grounds by Pantin (1931 *a*).

It is of interest, therefore, to inquire into the modifications, if any, which the ionic regulation of *Carcinus* undergoes when the animal, under the influence of a hypotonic environment, initiates osmotic regulation. It is known that the steady state attained in dilute sea water is characterized by an excretory rate that is greater than the normal (Nagel, 1934). Admittedly the urine, being isotonic with the blood, has a lower salt content than that produced in full-strength sea water. But as the fall in the concentration of the blood on transference from full-strength to half-strength sea water is only about 25 %, and the increase in the rate of urine production is about 70 %, the rate of salt excretion shows a net increase. There is also, in these circumstances, a considerable rise in the concentration gradient of sodium, potassium and chloride across the gill surface, and consequently a greater loss of these ions by diffusion. For two reasons, therefore, the rate of salt loss, and hence also the rate of salt absorption, must be greater in dilute media. Increased salt absorption and increased excretion should obviously, on the theory of ionic regulation suggested here, lead to an increase in the divergence of the ionic ratios of the blood from those of the external medium. Some analyses which I have made of the blood of crabs that had reached a steady state in 67 % sea water show that this takes place, and afford some corroborative evidence for the theory. The analyses are set out in tables 9 and 10. It should be noticed that the concentrations of magnesium and sulphate in the blood are reduced, not only relative to the other ions, but also relative to their concentration in the medium, even though this is only two-thirds of the normal. Such a reduction can hardly be of any functional significance, and is most easily interpreted as an inevitable consequence of osmotic regulation. These relations between osmotic and ionic regulation are illustrated graphically in figure 7.

TABLE 9. COMPOSITION OF THE BLOOD OF *CARCINUS* KEPT IN TWO-THIRDS SEA WATER. THE CONCENTRATIONS ARE EXPRESSED AS PERCENTAGES OF THE CONCENTRATION IN THE EXTERNAL MEDIUM, CALCULATED ON A WATER BASIS

	Concentration of external medium (g. Cl per l.)	Na	K	Ca	Mg	Cl	SO_4
	12·70	140·9	147·2	—	31·0	127·1	57·3
	13·35	134·0	128·7	—	35·7	120·4	42·2
	13·25	121·2	—	140·2	28·2	126·0	30·7
	12·63	146·3	149·3	133·7	30·3	132·6	63·3
	13·31	130·1	—	127·0	—	120·0	38·4
Mean	12·89	134·5	142·7	133·6	31·3	125·2	46·4

TABLE 10. AVERAGE COMPOSITION OF THE BLOOD OF *CARCINUS* IN FULL-STRENGTH AND IN TWO-THIRDS SEA WATER. CONCENTRATIONS ARE IN MG. PER G. WATER

	Na	K	Ca	Mg	Cl	SO_4
Sea water:						
Medium	10·68	0·386	0·408	1·287	19·23	2·68
Blood	11·84	0·467	0·517	0·460	19·19	1·53
Two-thirds sea water:						
Medium	7·15	0·259	0·274	0·862	12·89	1·80
Blood	9·62	0·369	0·366	0·270	16·11	0·835

The changes in the water balance which accompany osmotic regulation are rather more obscure. A good deal of confusion has been brought about by classing together under the general term "osmotic regulation" two processes which are fundamentally different: first, the attainment and maintenance of a steady state characterized by hypertonicity of the blood, and secondly, temporary measures during the transition period, of which the purpose is usually to prevent undue swelling before the permanent regulatory processes are under way. To the second class belongs the greater part of the osmoregulatory activity of certain animals, such as *Gunda* (Weil and Pantin 1931; Beadle 1934) and *Nereis diversicolor* (Schlieper 1929 *a*; Beadle 1931, 1937; Ellis 1937, 1939). This latter species is able to maintain a hypertonic body fluid indefinitely in dilute media, but its power of rapidly accommodating itself to changes in salinity without too much swelling is far more important for its survival under normal conditions. With regard to *Gunda*, it has yet to be shown that hypertonicity can be permanently maintained.

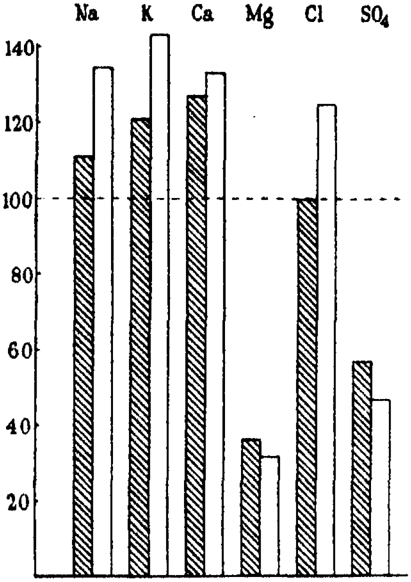

FIGURE 7. Illustrating the composition of the blood of *Carcinus*, when living in full-strength sea water (shaded columns) and 67 % sea water (unshaded columns). The concentration of each ion is expressed as a percentage of its concentration in the external medium. On transference from full-strength to dilute sea water the relative concentrations of Na, K, Ca and Cl rise (though their absolute concentration is of course diminished), while those of Mg and SO_4 fall.

In *Carcinus* both aspects of osmoregulation seem to be important. Bethe (1934) and Bethe, von Holst and Huf (1935) came to the conclusion that the rise of internal pressure which resulted from osmotic absorption of water was the stimulus to regulation, and that it was truer to speak of *Carcinus* as homoiobar than as homoiosmotic. Huf (1936), however, was able to show that the attachment of a wide sea-water manometer to a crab (by which means pressure changes were minimized) did not prevent the maintenance of hypertonicity when the medium was diluted. It is probable that the effects of pressure are very important in stimulating an increase in the rate of urine production during the initial transitional period, but that once the steady state has been established the blood pressure returns to normal. In the same paper Huf showed that for the first 3 hr. after a crab had been immersed in 50 % sea water urine is produced at a rate equivalent to almost 40 % of the body weight per 24 hr. This is certainly not maintained; so it would seem that the steady state is characterized by a reduction, though perhaps not a very large one, in the permeability of the gills to water. This point, as it concerns osmotic regulation alone, and has no effect on the ionic ratios in the blood, need not be pursued further. Enough has been said to make it plain that much, if not all, of the permanent part of osmoregulation is brought about by an intensification of the activities to which ionic regulation must be ascribed.

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DESCRIPTION OF PLATE 7

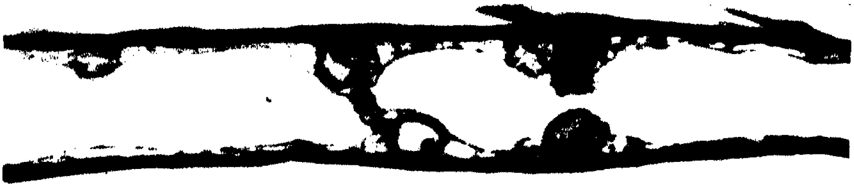
All photographs are of sections 5 μ thick of material fixed in Bouin's fluid, and stained in iron-haematoxylin and orange G. Magnification: 800.

FIGURES 1 and 2. Transverse section of part of two gill leaflets of *Maia squinado*. The typical appearance is seen in Fig. 2: epicuticle well developed and masking the chitinous layer; syncytial epithelium so thin as to be almost invisible, except in the region of the nuclei; cytoplasm hyaline. Fig. 1 is inserted to show a "pillar-cell" junction between the two sides of the lamella across the blood lacuna. These junctions are not abundant.

FIGURE 3. Longitudinal section of part of a gill filament of *Homarus vulgaris*. The space at the top of the photograph is the outside world: that at the bottom the blood lacuna in the centre of the filament. The distinction between the darkly staining epicuticle and the thicker and paler chitinous layer is most clearly seen on the right. Below the cuticle is the syncytial epithelium, largely hollowed out by subsidiary blood lacunae.

FIGURE 4. Transverse section of the central region of a gill leaflet of *Eriocheir sinensis*. No cuticle can be seen. The epithelium is everywhere at least 2·5 μ thick, with granular cytoplasm. A strand of connective tissue runs down the centre of the leaflet.

FIGURE 5. Transverse section of part of a gill leaflet of *Carcinus maenas*. Cuticle as in *Maia*, but somewhat thicker; epithelium much better developed, with frequent "pillar-cell" junctions.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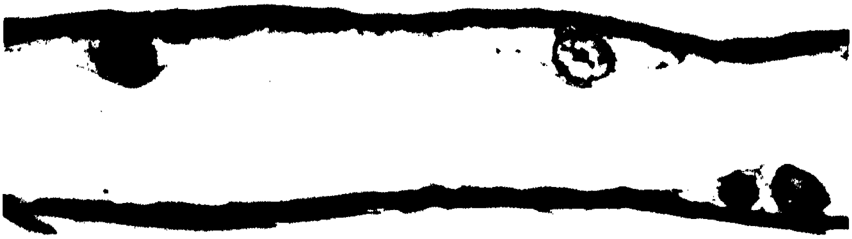
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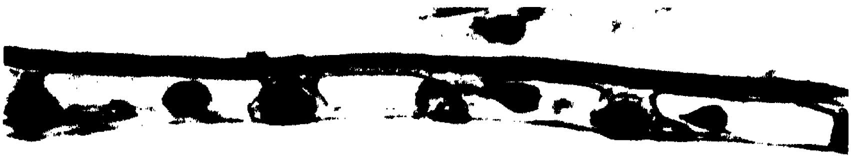
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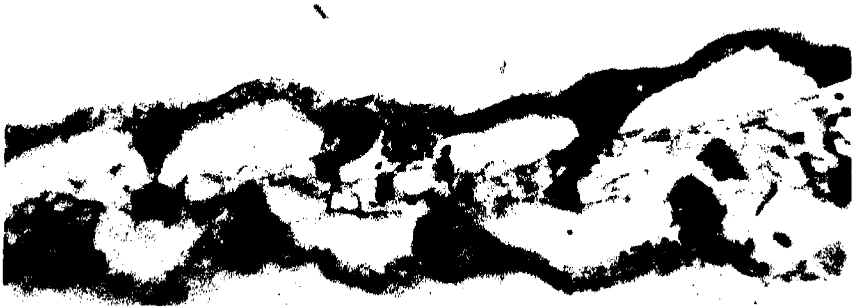
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On the function of chlorocruorin*

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1. The oxygen consumption of *Sabella* decreases soon after the concentration of dissolved oxygen in the sea water falls below the value corresponding to air saturation both at 10 and 17° C. Above air saturation at 17° C there is no significant rise in oxygen consumption.

2. The oxygen consumption of *Sabella* whose chlorocruorin has been converted to carboxychlorocruorin is lower than that of normal worms. This is the case at air saturation of the water and at oxygen concentrations below air saturation both at 10 and 17° C.

3. The fall in oxygen consumption of the animals after treatment with carbon monoxide is not due to an action of the latter on cell enzymes.

4. It follows that chlorocruorin functions as an oxygen carrier in *Sabella* at all temperatures and oxygen pressures to which the animals are subjected in nature.

1. INTRODUCTION

Chlorocruorin is a pigment closely related to haemoglobin. It is found dissolved in the blood plasma of certain marine polychaete worms. A series of papers has dealt with the properties of chlorocruorin, and with the blood circulation and metabolism of sabellid worms possessing this pigment (Fox 1926, 1932, 1933, 1934, 1938†; Roche and Fox 1933; D. W. Ewer, in the press). Although it can be seen from these papers that chlorocruorin probably has a respiratory function, this has not yet been demonstrated. The present investigation was undertaken in order to discover whether chlorocruorin actually does function as a respiratory pigment.

The method adopted depends on the fact that chlorocruorin forms with carbon monoxide a compound analogous to carboxyhaemoglobin (Fox 1926). It is therefore possible to eliminate any oxygen-carrying property of the pigment by treatment with carbon monoxide, and to compare the respiration of worms so treated with that of normal worms.

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* A preliminary notice of the contents of this paper has been published (Fox 1940).

† It is stated on p. 559 of this paper that *Spirographis* and *Sabella* removed from their tubes are incapable of making new ones; Bobin (1939) has now shown that this is not the case. There is also an error on p. 566: the figures on line 6 should be 5·8 ± 1·1 : 1 and on line 8 450/5·8 = 78; this typographical error does not affect the calculation.

If chlorocruorin does act as an oxygen carrier, it may be that the pigment does not function when a worm is in air-saturated water, but only comes into action in water deficient in oxygen, the amount of oxygen in simple solution in the worm's blood being sufficient for its needs in air-saturated water. It was therefore necessary to study the effect of carbon monoxide on the oxygen consumption of the animals at a series of different concentrations of oxygen dissolved in the sea water.

All the experiments were made with *Sabella pavonina* Savigny, the worms having been removed from their tubes. It would have been preferable to study animals in their tubes with their crowns expanded, as this would have been closer to natural conditions. This was not possible, however, because the water in the experimental vessel cannot be stirred adequately without disturbing the animal and frequently making it withdraw its crown into its tube, thus causing great irregularities in respiratory rate. In this respect *Sabella* behaves differently from the nearly related *Spirographis spallanzanii* Viviani, whose crown remains expanded while the water is stirred (Fox 1938, p. 562).

After *Spirographis* has been removed from its tube, its metabolism falls and continues to decline progressively for at least 24 hr. (Fox 1938). Preliminary experiments showed us that in *Sabella* too the oxygen consumption is less on the day after worms have been removed from their tubes than it is immediately after removal. Four tests made it clear however that in our experiments, which were all begun with worms as soon as they had been removed from their tubes, the metabolism did not appreciably diminish during the experimental period, for when the animals were replaced in normal sea-water after their respiration had been measured for 5 hr. in water of diminishing oxygen content they showed complete recovery. There was therefore no need to make a correction for progressively declining oxygen consumption due to the worms being tubeless.

2. THE EFFECT OF AVAILABLE OXYGEN ON THE RESPIRATION OF *SABELLA*

As a first step in the investigation a study was made of the relation between the oxygen consumption of *S. pavonina* and the concentration of dissolved oxygen in the sea water, without any treatment of the worms with carbon monoxide. This was done in 1935 in the Zoology Department of University College, London, with animals sent from Plymouth, and we wish to tender our sincere thanks to Professor D. M. S. Watson, F.R.S., for his hospitality.

In each of these preliminary experiments a single worm was put into a closed flask of known volume, completely filled with sea water which had a desired concentration of dissolved oxygen. The flask was immersed in a water thermostat at 17° C. The flask was of such a size that the worm reduced the oxygen content of the water by 10–15 % of the original value in 1–2 hr. During the experiment the flask was twisted by hand every 5 min. to circulate the water in it. Water was used which had a content of dissolved oxygen greater or less than that of sea water in equilibrium with air. Four series of experiments were made, namely one series with a mean oxygen concentration of 10·2 c.c. per litre (extreme values in separate experiments 10·8 and 9·5), or nearly twice the normal oxygen content of sea water at 17° C, and three series with lower oxygen concentrations than that of sea water in equilibrium with air. These three series had mean oxygen concentrations of 4·4, 2·9 and 1·3 c.c. per litre respectively (extreme values 4·9–4·0, 3·1–2·7, 1·6–1·1). The water was prepared as follows. It was given approximately the desired concentration of dissolved oxygen by first equilibrating it with air and then bubbling oxygen or nitrogen through a measured volume of the water at a known rate for a previously determined time, the rate of flow of the gas being measured with Beckett's (1917) glass meter. The oxygen content of the experimental water was then analysed, and at the end of the experiment another water sample was removed from the experimental bottle for analysis. Each analysis was done in duplicate on 2·5 c.c. of water by a simplified variation of Nicloux's modification of the Winkler method (Fox, Wingfield and Simmonds 1937, p. 212). The oxygen concentration in the water during the experiment was taken as being the mean of the initial and final values. After each experiment the worm was dried on filter paper and weighed.

The results of the experiments, expressed as cu.mm. oxygen consumed per gram wet weight of worm per hour, are given in table 1 and figure 1. It is clear that the oxygen consumption decreases as the available oxygen falls below the amount present in sea water equilibrated with air. There is apparently a slight increase in respiration in water with a higher oxygen content, but the number of experiments was too small to show that this increase is significant, for, while table 1 shows that the difference between the mean oxygen consumption values at oxygen concentrations of 10·2 and 4·2 c.c. per litre is 10·7, the standard error of this difference is 6·2.

It is noticeable that there are great individual variations in oxygen consumption. This has been found before with other invertebrates (Fox 1936). Differences in muscular activity may to a certain extent be responsible for such variations, although it is not obvious that muscular activity in one individual *Sabella* is much greater than in another, but the variations are probably due in part to differences in the size of the animals and also to differences in the relative sizes of their crowns. It is known that in various poikilothermal animals the rate of metabolism per unit weight is greater the smaller the animal; the literature has recently been given by Wingfield (1939, p. 105). Moreover, in *Spirographis*, which is nearly related to *Sabella*, larger animals have a slower rate of blood vessel pulsations (Fox 1938, p. 557). In the four series of experiments on *Sabella* summarized in table 1 the correlation coefficients between weight and oxygen consumption were respectively −0·43, −0·05, −0·64 and −0·65. Thus on the whole the larger animals tend to have a lower oxygen consumption.

TABLE 1. OXYGEN CONSUMPTION OF *SABELLA PAVONINA* AT 17° C AT VARIOUS CONCENTRATIONS OF DISSOLVED OXYGEN

Mean oxygen concentration c.c./l.	Oxygen consumption cu.mm./g. (wet)/hr.	
	In each experiment	Mean and S.E.
10·2	79, 104, 68, 79, 65, 79, 78, 93, 108, 115, 91, 70, 80	85·3 ± 4·4
4·4	62, 66, 88, 67, 95, 40, 62, 65, 92, 83, 107, 87, 58, 87, 83, 78, 48	74·6 ± 4·3
2·9	86, 48, 61, 63, 48, 80, 58, 53, 44, 79, 48, 56, 84	62
1·3	37, 57, 31, 35, 40, 55, 41, 28, 30, 45, 48, 38, 51	41

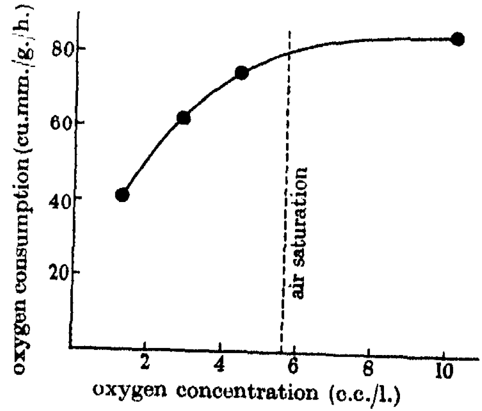

FIGURE 1. Rates of oxygen consumption at 17° C of *Sabella pavonina* at various concentrations of dissolved oxygen.

The crown of *Sabella* is largely responsible for the oxygen intake of the animal (Fox 1938), and the size of the crown relative to that of the body varies considerably in different individuals, small crowns being probably in a state of incomplete regeneration. No animals with very small crowns were used in the experiments; nevertheless there are inevitable individual differences in the ratio of crown to body size. In the two series of experiments at 10·2 and at 2·9 c.c. oxygen per litre the body and crown of each worm was weighed separately, and a positive correlation was found between the ratio of crown to body and the magnitude of oxygen consumption. In the first series the correlation coefficient was +0·35, in the second +0·72. Thus part of the individual differences in oxygen consumption were probably due to varying sizes of the crowns.

3. THE EFFECT OF CARBON MONOXIDE ON RESPIRATION OF *SABELLA*

For the main experiments on the function of chlorocruorin, which were made in Birmingham with *S. pavonina* sent from Plymouth and from Millport, a different method was adopted for the measurement of oxygen consumption, for the following reason. It appears that certain aquatic animals (planarians and caddis larvae) whose oxygen consumption is dependent on the available oxygen may, to a certain extent, adapt themselves to conditions of oxygen-lack if given time. When such an animal is put *suddenly* into water of diminished oxygen content, its respiration falls, but if the oxygen content of the water in which the animal is living is only *slowly* brought down to the same value, then the animal's respiration does not fall so much (Hyman 1929; Hiestand 1931). It is possible, therefore, that when *Sabella*, retracted in its tube, slowly reduces the available oxygen its oxygen uptake does not diminish so much as in the experiments already described. An apparatus was therefore designed in which the worm slowly reduces the oxygen in the experimental bottle by its own respiration, water samples being withdrawn from the bottle at intervals for analysis. Figure 2 shows the bottle. It is a conical glass flask of about 350 c.c. capacity with a three-way

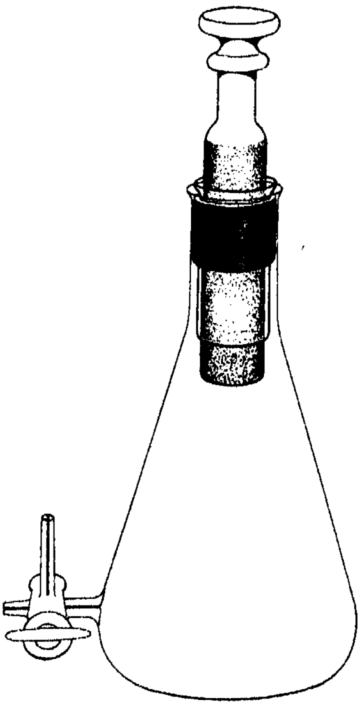
FIGURE 2. Respiration flask having a three-way tap and a stopper formed by a glass syringe with its barrel cut short inserted into rubber tubing (shown black). (Drawn by Dr D. P. Pielou.)

tap of capillary bore fixed near the base. The flask is closed by a glass syringe whose barrel has been cut short so that it has no nozzle. The joint between the truncated barrel of the syringe and the neck of the flask is made airtight with rubber tubing. After a worm has been put into the water in the flask for an experiment, the flask is closed without including any air bubbles, and after the tap has been opened the plunger is allowed to descend to a certain mark by which the volume of water in the flask is known. The tap is then closed. When water is withdrawn through the tap for analysis during an experiment the plunger slides down by a corresponding amount, and it is thus possible to take a number of water samples at intervals without admitting air. The diminished volume of water in the flask after each sample has been withdrawn is allowed for in the calculation.

For each experiment the water was given approximately the desired oxygen content in the manner described above. The experiments at 17° C were all started with air-saturated water. Some of those at 10° C were begun at concentrations of oxygen below air saturation* because with the slower respiration at the lower temperature the time taken for the animals to reduce the oxygen content of the water from air saturation to about 1 c.c. per litre is very long.

Before and at intervals during an experiment the oxygen content of the water was determined in 1·5 c.c. samples by the syringe-pipette method of Fox and Wingfield (1938). For withdrawal of the water sample the nozzle of the syringe pipette, containing some manganous chloride solution, is connected to the horizontal outlet of the three-way tap with rubber tubing, the passage from the horizontal to the vertical outlet of the tap being open. The plunger of the syringe pipette is then pressed home, the tap being thus filled with manganous chloride solution. After that the cock is turned so that the flask is in connection with the syringe pipette, the vertical outlet of the tap being closed, and the water sample is withdrawn into the syringe for analysis. A possible error due to the manganous chloride solution in the tap replacing part of the measured volume of water to be analysed was found to be negligible: water taken into the syringe pipette through the tap and water from the top of the flask showed the same oxygen content on analysis.

During an experiment the flask was attached in a water thermostat to a wheel rotating slowly on a horizontal axle. This resulted in the water inside the flask being gently and continuously circulated.

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* This procedure was justified since the experiments at 17° C with the flask shown in figure 2 proved that there is no respiratory adaptation even when the oxygen is reduced gradually (*vide infra*).

The method of treating worms with carbon monoxide was as follows. Sea water was prepared containing approximately 1 c.c. of carbon monoxide and 5 c.c. of oxygen per litre. The worm to be used was placed in this water in its tube, and kept in the dark for 3 hr. It was necessary to use as high a concentration of carbon monoxide as this because lower concentrations were found not always to be effective. The volume of water used was large, so that during the 3 hr. its oxygen concentration was scarcely reduced; in other words the relative pressure of carbon monoxide was never significantly greater than 1/5.

After an experiment the animal was weighed as usual, and then some blood was withdrawn, diluted and examined spectroscopically to ascertain whether the chlorocruorin had all been converted to the carboxy-compound. This is easily done, since oxychlorocruorin and carboxychlorocruorin have intense absorption bands, whereas reduced chlorocruorin has not (Fox 1926). Oxychlorocruorin can be reduced by sodium hydrosulphite, but carboxychlorocruorin cannot; consequently if the bands do not become appreciably weaker when the salt is added to the blood, almost all of the pigment must be in the state of carboxychlorocruorin.

The experimental results are given in tables 2 and 3, and in figures 3 and 4. It should be remembered that in the preliminary experiments, summarized in table 1, there was a single oxygen-consumption value, at a given oxygen concentration, in each experiment. In the experiments now being described, however, water samples were withdrawn from the bottle at intervals, the oxygen content of the water having fallen, owing to the worm's respiration, between the successive withdrawals of water for analysis. Thus in each experiment there was a number of oxygen-consumption values, corresponding to the successive diminishing oxygen concentrations. The oxygen concentration in the water corresponding to each of these oxygen-consumption values was taken as the mean of the oxygen concentrations determined at the beginning and end of the interval in question. In tables 2 and 3 the oxygen-concentration values of all the experiments are grouped in classes differing by 0·5 c.c. per litre, but no attempt is made to distinguish the series of values belonging to separate experiments.

It is clear from figures 3 and 4 that the oxygen consumption of the worms diminishes with falling oxygen content of the water. The upper curve in figure 3, for animals at 17° which had not been treated with carbon monoxide, is comparable with the preliminary experiments of figure 1. It is evident from figure 3 that although the worms themselves reduced the oxygen content of the water slowly, yet there was no adaptation in oxygen

TABLE 2. OXYGEN CONSUMPTION OF *SABELLA PAVONINA* AT VARIOUS CONCENTRATIONS OF DISSOLVED OXYGEN

Temp. °C	Oxygen concentration c.c./l.	Oxygen consumption cu.mm./g. (wet)/hr. Separate values	Mean and S.E.
17	5·5–5·1	84, 64, 57, 51, 61, 72, 60, 67, 38	62
	5·0–4·6	71, 24, 92, 57, 56, 109, 39, 54, 56, 48	60·6 ± 7·8
	4·5–4·1	64, 37, 55, 93, 55, 47, 65, 54, 70	60·0 ± 5·3
	4·0–3·6	66, 57, 64, 92, 39, 53, 45, 41	57
	3·5–3·1	61, 48, 58, 62, 84, 43, 34, 52, 59, 87, 61	59
	3·0–2·6	57, 51, 44, 55, 68, 27, 38, 34, 52, 47, 35, 54	47
	2·5–2·1	35, 48, 55, 50, 44, 44, 44, 46, 33	44
	2·0–1·6	60, 43, 32, 48, 50, 11, 37, 33, 37	39
	1·5–1·1	33, 32, 14, 29	27
10	5·5–5·1	41, 42, 22, 63, 55, 52, 45, 23, 40	43
	5·0–4·6	44, 53, 54, 40, 41, 48, 53, 55, 35	47
	4·5–4·1	35, 47, 33, 30, 30, 48, 53, 23, 32, 38	37
	4·0–3·6	57, 38, 43, 38, 31, 38, 34, 35, 23, 33, 37	37
	3·5–3·1	26, 32, 35, 37, 32, 35, 46, 20, 15, 40, 28, 31, 25, 30, 39	31
	3·0–2·6	33, 36, 38, 27, 28, 33, 50, 28, 29, 47, 29, 53, 34, 29, 31, 49, 20	35
	2·5–2·1	36, 29, 26, 29, 36, 38, 31, 35, 18, 48, 39, 31, 29, 31	33
	2·0–1·6	24, 27, 10, 35, 30, 21, 23, 32	25
	1·5–1·1	12, 28, 39, 19, 20, 18, 15	22
	1·0–0·6	10, 18, 13, 10, 14, 9, 9	12

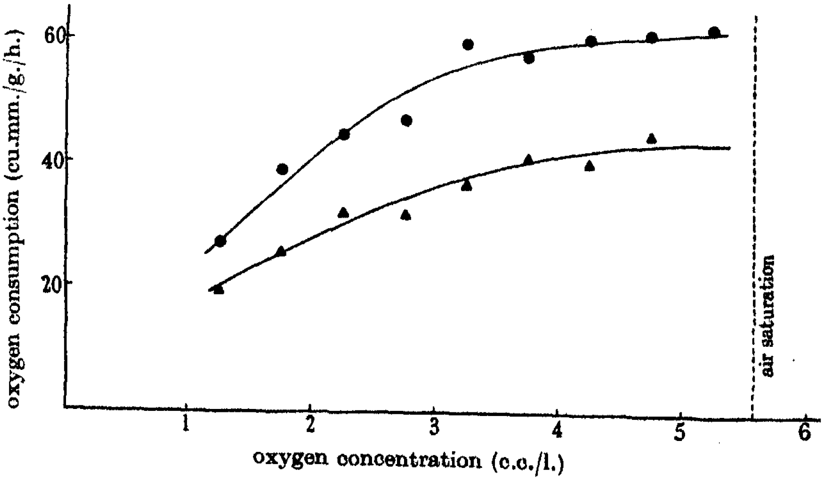

FIGURE 3. Rates of oxygen consumption at 17° C of *Sabella pavonina* at various concentrations of dissolved oxygen; ● normal animals, ▲ animals with carboxy-chlorocruorin.

consumption; the metabolism fell with the decrease in available oxygen just as it did in the preliminary experiments.

TABLE 3. OXYGEN CONSUMPTION OF *SABELLA PAVONINA* AT VARIOUS CONCENTRATIONS OF DISSOLVED OXYGEN AFTER TREATMENT WITH CARBON MONOXIDE

Temp. °C	Oxygen concentration c.c./l.	Oxygen consumption cu.mm./g. (wet)/hr. Separate values	Mean
17	5·0–4·6	52, 50, 47, 23, 38, 34, 43, 36, 39, 37, 36, 47, 39, 66, 60, 67	45
	4·5–4·1	40, 56, 39, 37, 52, 42, 31, 29, 41, 37, 35	40
	4·0–3·6	49, 49, 34, 33, 39, 41, 31, 33, 56	41
	3·5–3·1	28, 28, 29, 59, 32, 49, 34, 43, 31, 34, 29, 56, 31, 24	36
	3·0–2·6	22, 26, 37, 44, 43, 20, 36, 34, 27, 25, 31, 33	31
	2·5–2·1	27, 30, 24, 31, 27, 46, 37, 32, 32, 30	32
	2·0–1·6	30, 19, 17, 29, 21, 43, 12, 30, 31, 25, 24	26
	1·5–1·1	17, 19, 17, 21, 15, 26	19
10	5·5–5·1	19, 21, 35, 24, 19	24
	5·0–4·6	20, 21, 26, 26, 16, 32, 23, 31, 18, 23	24
	4·5–4·1	20, 14, 18, 19, 23, 32, 18, 23, 20, 21	21
	4·0–3·6	16, 21, 19, 24, 22, 18, 16, 19	19

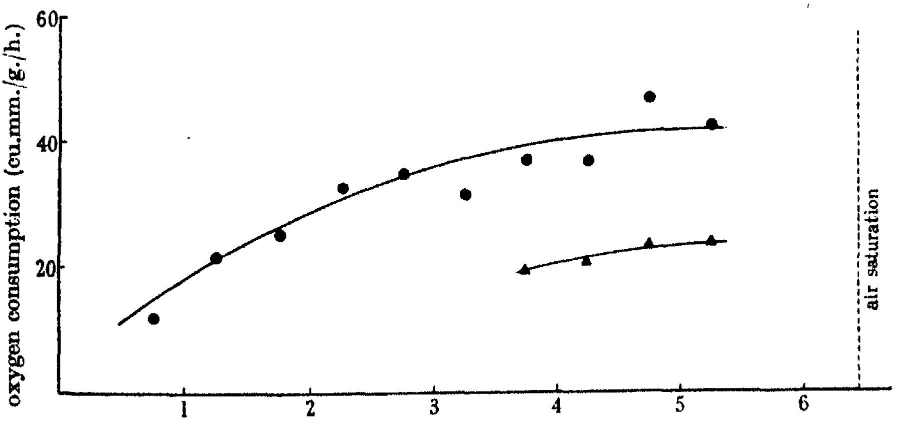

FIGURE 4. Rates of oxygen consumption at 10° C of *Sabella pavonina* at various concentrations of dissolved oxygen; ● normal animals, ▲ animals with carboxychlorocruorin.

It is noticeable, however, that the oxygen consumption values at 17° in figure 3 are lower than those in figure 1. It is possible that the gentler agitation of the water in the later technique caused less muscular contraction of the worms, but the difference is probably due mainly to a size difference between the animals employed. In the preliminary experiments of figure 1 the mean wet weight per animal was 1·7 g., in the experiments of figure 3 it was 4·5 g.

Figures 3 and 4 show clearly that both at 17 and at 10° C, and at all oxygen concentrations used, the respiration of worms treated with carbon monoxide is less than that of normal worms. Thus at both temperatures respiration diminishes considerably when the chlorocruorin is eliminated.

A comparison of figures 3 and 4 shows that carbon monoxide caused a greater diminution in oxygen consumption at 10 than at 17° C. This is probably to be attributed again to size differences of the animals. The average weights of the worms used were as follows: at 17° C, untreated animals 4·5 g., with carbon monoxide 4·8 g.; at 10° C, untreated 1·8 g., with carbon monoxide 6·2 g. The differences in weights at 10° C were due to exigencies of collection.

4. Possible sources of error

(1) For the successful operation of the Winkler method of estimating dissolved oxygen it is essential that the animal should not liberate reducing substances which might give a fallaciously low value for the oxygen content of the water. The most frequent sources of error are nitrite and ferrous iron (Allee and Oesting 1934), but tests showed that these substances were not present in our experiments.

(2) Organic substances secreted by an animal might cause an error in the oxygen estimation by absorbing iodine during the titration. That this is not the case was shown as follows: when the sodium thiosulphate solution was standardized against sodium iodate which had been diluted with sea water in which a worm had lain, instead of distilled water, the result was the same as when distilled water was used for the dilution.

(3) It has been shown by Yonge (1937) that some corals secrete much mucus, which absorbs oxygen. *Sabella* produces some mucus, and this might therefore be a source of error in oxygen determinations. The possibility of this error was tested by cutting worms into pieces, which stimulates mucus secretion. After bleeding had stopped, the pieces were washed and left in an experimental flask for 2 hr., in which time they produced at least as much mucus as is normally present in an experiment. During the two hours subsequent to the removal of the pieces of worms from this water, its oxygen content was found to fall only at the rate of 0·014 c.c. per

litre per hour. This is comparable with the reduction in the oxygen content of ordinary sea water in an experimental flask containing no worm, namely, 0·012 c.c. per litre per hour, which is possibly due to absorption by the rubber tubing holding the syringe stopper in place. These two factors were regarded as small enough to neglect, especially as the work was of a comparative nature and the effect must be approximately the same in each experiment.

(4) The experiments on worms treated with carbon monoxide, summarized in table 3, were made in darkness, since light dissociates carboxychlorocruorin (Fox 1926), whereas the experiments on untreated worms (table 2) were made in dim daylight. Part of the difference in oxygen consumption between treated and untreated worms might therefore have been due to a difference in illumination, not to the effect of carbon monoxide. To test this possibility additional experiments were made at 17° C with untreated worms in darkness. Eleven experiments at an oxygen concentration of 5·0–4·6 c.c. per litre gave a mean oxygen consumption of 55·0 ± 2·6 c.c. per gram wet weight of worm per hour. The difference between this and the corresponding value of 60·6 ± 7·8 given in table 2 is 5·6 and the standard error of this difference is 8·2. Twelve other experiments in darkness at an oxygen concentration of 4·5–4·1 c.c. per litre gave a mean oxygen consumption of 55·6 ± 2·9 c.c. per gram wet weight of worm per hour. The difference between this and the value of 60·0 ± 5·3 in table 2 is 4·4 and the standard error of this difference is 6·0. In neither case therefore is the difference between the oxygen consumptions in light and darkness significant.

(5) It has been shown by Fox (1933) that carbon dioxide in solution causes an inhibition of the contractions of the blood vessels of *Sabella*, but this only takes place when there is a considerable amount of dissolved carbon dioxide present, the threshold being about pH 6·0. The pH of the water at the end of several experiments was therefore determined, and found to be 8·0. This is far above the threshold, so that the carbon dioxide liberated during the course of an experiment was not sufficient to introduce an error due to inhibition of the blood vessel contractions. Moreover, at the end of an experiment the blood vessels of the crown, which are the only ones easily observable in large worms, could be seen contracting normally.

(6) Carbon monoxide not only unites with chlorocruorin but also inhibits cellular respiration (Warburg 1926; Keilin 1929). Before we can conclude, therefore, that the diminution in oxygen consumption of *Sabella* after treatment with carbon monoxide is due to the elimination of the respiratory function of chlorocruorin, it must be shown that carbon monoxide, at the

pressures used, has no effect on cellular respiration. It was *a priori* unlikely that the carbon monoxide had any such effect in our experiments because in other cases so far studied the oxidase is only affected by high partial pressures of the gas, when the relative pressure of carbon monoxide to oxygen is greater than unity. Nevertheless, it was thought necessary to test the possibility of this error experimentally. The influence of carbon monoxide on the tissue respiration of *Sabella* was therefore investigated.

A Barcroft differential manometer was used, the flasks of which were fitted with side taps so that a stream of gas could be passed through them. The tissue was prepared as follows. The middle region of the body of a worm was opened dorsally and the gut removed, after which the body wall was cut into slices about 1 mm. in thickness. This relatively great thickness is allowable owing to the slow rate of respiration of the tissue. Warburg's (1923) formula for the maximum thickness which will allow oxygen to reach the cells in the interior of the tissue gives a value of *ca.* 1·5 mm. for *Sabella*, assuming that the rate of diffusion of oxygen into the tissue is the same as that found by Krogh (1919) for muscle*. After washing, the slices were put into one of the experimental flasks with 3 c.c. M/1000 succinic acid in sea water, the pH of the solution having been adjusted to that of normal sea water. This solution was found to give a more uniform oxygen uptake than sea water alone or sea water + 1 % glucose. The experimental temperature was 17° C and the rate of shaking 130 per min. with an excursion of 5 cm. A further increase in rate of shaking did not accelerate the rate of respiration.

During the experimental period there was a slight continuous falling off in the rate of respiration; the following procedure was therefore adopted. After the tissue and solution had been put into one flask, and solution alone into the other, air was passed through both flasks for 20 min., and then an interval of 10 min. was allowed for temperature and pressure equilibration. During all this period the apparatus was shaken. The respiration of the tissue was then measured for 1 hr. After that a gas mixture containing 4·2 % carbon monoxide and 95·8 % air, which has the same relative pressure of carbon monoxide and oxygen, namely, 1/5, which was used for the whole worms, was passed through the flasks for 20 min., followed by 10 min. for equilibration. The respiration was then again measured for 1 hr. The whole experiment was done with the flasks in darkness. After the experiment the tissue was filtered off on a Gooch crucible, washed with distilled water, dried for 24 hr. at 105° C, and weighed. Another series of experiments was made which was exactly similar to the first except that air instead of the carbon monoxide mixture was passed through the flasks after the first hour's measurement of respiration.

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* Warburg (1923) and Dixon (1934) incorrectly quote Krogh as finding a diffusion constant of $1{\cdot}4 \times 10^{-5}$ at 38° C, whereas the temperature for which Krogh's results are given is 20° C.

TABLE 4. OXYGEN CONSUMPTION OF TISSUE SLICES OF *SABELLA PAVONINA* AT 17° C IN AIR AND IN CARBON MONOXIDE

First series			Second series		
	Oxygen consumption cu.mm./g.(dry)/hr.			Oxygen consumption cu.mm./g.(dry)/hr.	
Exp. no.	First hour, in air	Second hour, in carbon monoxide	Exp. no.	First hour, in air	Second hour, in air
1	459	419	12	586	523
2	578	482	13	524	392
3	566	489	14	503	395
4	574	447	15	508	431
5	547	444	16	582	368
6	462	462	17	430	430
7	427	377	18	507	419
8	430	327	19	637	505
9	464	410	20	493	493
10	584	479	21	629	490
11	449	396	22	532	436
Mean	504	430	Mean	539	444

As stated above, there was usually a decrease in the tissue respiration with time. In order to allow for this, the ratio, in the first series of experiments, of the respiration in the carbon monoxide mixture to that in air during the previous hour was compared with the ratio, in the second series, of the two successive hours' respiration in air. The results are given in table 4. The mean rate of respiration in carbon monoxide mixture, namely, 430 cu.mm. per gram dry* weight per hour, is 85 % of 504, the rate during the previous hour in air. In the second series of experiments 444, the rate during the second hour in air is 82 % of 539, the rate during the first hour in air. The two percentage decreases are thus almost identical, which shows that the carbon monoxide had no inhibiting effect on the tissue respiration.

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* These rates of metabolism can be compared with those of whole animals by employing the factor 5·8, the ratio of wet weight to dry weight.

5. Discussion

In a number of invertebrates there occur blood pigments which are presumed to be respiratory, but only in a few instances have they been shown to act in the same way as respiratory pigments do in the vertebrates.

It has been suggested that in certain invertebrates these pigments are not utilized at all when the pressure of oxygen in the environment is relatively high, since enough oxygen to meet the requirements of the animal can then be carried in simple solution in the blood: only at low environmental oxygen pressures would the pigments be used by the animals. In such a case, assuming the pigment to be haemoglobin, its conversion to the carboxy compound would cause no diminution in the animal's oxygen consumption until the external oxygen pressure had been reduced to a low value. This has been reported for the earthworm (Jordan and Schwarz 1920; Dolk and van der Paauw 1929), but experiments carried out in this Department and shortly to be published by Dr M. L. Johnson have failed to confirm their results*. The haemoglobin of *Arenicola* has a low loading tension (oxygen pressure for 95 per cent oxyhaemoglobin) (Barcroft and Barcroft 1924); it therefore takes up oxygen from the environment even when the latter contains very little dissolved oxygen. For this reason it has been concluded (Borden 1931) that the primary function of the haemoglobin of *Arenicola* is that of transporting oxygen during the period of lowered oxygen pressure to which the worms are probably subjected in their burrows at low tide. This suggested function for *Arenicola* haemoglobin, however, lacks experimental proof, and if the oxygen pressure in the respiring tissues were near zero, the oxyhaemoglobin, in spite of its low unloading tension (oxygen pressure for 50 per cent oxyhaemoglobin), would give up its oxygen to these tissues even at high oxygen pressures in the environment.

It has also been suggested that in some cases among the invertebrates a respiratory pigment does not act as a continuous oxygen transporter from surface to cells, but as a reservoir of the gas for use in times of oxygen shortage. Thus the amount of oxygen in the blood of *Arenicola* is sufficient to last the animal for about an hour at 10° C, and could therefore help to supply its wants while it is buried in mud at low tide and unable to obtain fresh supplies of oxygen (Barcroft and Barcroft 1924; Borden 1931). Van Dam (1938) points out that the ventilation movements of *Arenicola* in its mud tube under water are intermittent, and he concludes that the haemoglobin may act as a store of oxygen between successive ventilation periods.

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* A preliminary notice of these results has been published (Fox 1940).

In the gephyrean worm *Urechis caupo*, too, the oxygen combined with the haemoglobin is sufficient to last the animal for about an hour and is thought to act as a store both between ventilation periods and between tides (Redfield and Florkin 1931).

Our experiments have shown that in *Sabella* the oxygen consumption of worms treated with carbon monoxide is less than that of normal worms, at oxygen concentrations from air saturation down to a low value. The decrease is not due to a direct effect of carbon monoxide on the tissues of the animal, and must therefore be the result of the elimination of oxygen carriage by the chlorocruorin in the blood. The difference between the oxygen consumption of normal animals and of those treated with carbon monoxide gives a measure of the respiratory activity of chlorocruorin at the temperatures and oxygen concentrations studied, and shows that chlorocruorin functions as a respiratory pigment when *Sabella* is in air-saturated water as well as in water of lesser oxygen content.

This might have been anticipated from the fact that the oxygen consumption of the worms is dependent on the oxygen tension in the water: soon after the latter decreases the oxygen consumption falls, not only at 17° C, but also when the metabolism of the animals is lower, at 10° C. Since this is the case, it is to be presumed that the oxygen pressure in at least some of the tissues is very low (Krogh 1916), and therefore that the respiratory pigment will unload most of its oxygen on arrival at these tissues. Moreover, if the relatively high unloading tensions—18 mm. at pH 7·7 and 10° C, 27 mm. at pH 7·7 and 20° C—found by Fox (1932) for *Spirographis* chlorocruorin in diluted blood apply also to whole blood of *Sabella*, the pigment would easily give up its oxygen to the tissues even if the oxygen pressure in them were not zero. *Tubifex* is the only other annelid which has been shown to use its blood pigment when in water saturated with air (Dausend 1931).

It is probable that *Sabella* does not usually encounter conditions of serious oxygen shortage for any length of time. *Sabella* lives in fairly shallow water, from low tide-mark down to about 100 m. (McIntosh 1922), and the oxygen concentration of sea water at the latter depth is moderately high, being 4 to 7 c.c. per litre down to 500 m. in the Atlantic N. lat. 30–60° (Johnstone 1923). Nevertheless in some places *Sabella* is uncovered at low tide; for instance, during lowest summer spring tides at Baltimore, Co. Cork*, some individuals were found to be exposed for 4 hr. In these conditions there is probably a deficient oxygen supply in the tubes. It is improbable, however, that the chlorocruorin is of much importance as an oxygen store. The total amount of oxygen carried by the blood of *Spirographis* is 6 cu.mm. per gram wet weight of worm (Fox 1934) and the value for *Sabella* is not likely to be very different. This would only be sufficient at 10° C to maintain the oxygen consumption of the worm for 9 min. at its normal level which is 43 cu.mm. per gram per hour (table 2). At 17° C, with an oxygen consumption of 62 cu.mm. per gram per hour, the oxygen in the blood would last only 6 min. On the other hand, soon after a worm is enclosed in its tube at low tide its oxygen consumption presumably begins to diminish owing to the lesser amount of oxygen available from outside. The oxygen in the blood might therefore last rather longer than 6 or 9 min., but not for any considerable length of time.

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* Our thanks are due to Professor L. P. W. Renouf, Director of the Loch Ine Biological Station where these observations were made.

We come to the conclusion then that the chlorocruorin in the blood of *Sabella* is of comparatively little importance as an oxygen store and that its main function is that of transporting oxygen.

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Observations on the structure, development and electrical reactions of the internal ear of the shaker-1 mouse (*Mus musculus*)

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(Plates 8–12)

A comparative study has been made of the embryology, anatomy and electrical reactions of the cochlea in the normal and shaker-1 mouse between the ages of 9 hours and 329 days.

Cochlear degeneration in the latter is initiated after birth in a fully formed organ, and is associated with marked changes in its electrical reactions.

In 1910 van Lennep published a little-known account of the post-foetal development of the internal ear of the Japanese waltzing mouse (a form of *Mus bactrianus*). He found that the pathological changes were confined to the cochlea and consisted of degeneration of Corti's organ and the stria vascularis, with later atrophy of the spiral ganglion. He further established that up to 5 days after birth the organ of hearing showed no morphological abnormalities, although at this stage changes in the vessels of the stria vascularis could be recognized. Ten days after birth changes in the hair cells of Corti's organ appeared and were thereafter progressive with age. The normal post-foetal development of Corti's organ is in any case not completed until about the end of the second week, and these findings are thus in fair accordance with the observation by Yerkes (1907) of acoustic reactions in some of his waltzers for a limited period during the third week, although this was not confirmed by Gates (1926).

Morphologically, at any rate, it would thus seem that the pathological sequence of events and the resulting deafness is enacted after birth and cannot therefore be regarded as congenital.

The shaker mutation (symbol *sh*-1) was described by Lord and Gates

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(1929). It resembles in many ways the waltzing mouse. Choreic head movements, from which it takes its name, and deafness are constant features. Some animals also run in circles, though not so persistently nor in such narrow circles as the Japanese waltzing mouse. The choreic head movements are fully developed at the age of 12–14 days, though some animals show them earlier. Young shakers, according to Lord and Gates, have a normal sense of hearing. Deafness develops rapidly within a few days, starting at the age of 22–30 days. Shaker is fully recessive; heterozygotes are completely normal in their behaviour and retain the sense of hearing throughout life. The same applies to animals heterozygous for the gene of waltzing. A very unusual situation, however, arises in animals which are heterozygous for both shaking and waltzing ($+/sh\text{-}1;\ +/v$); these, though they neither shake nor run in circles, have a tendency to become deaf from the age of about 3 months onwards. Thus, the two genes, though they are not allelomorphic to each other and are carried in different chromosomes, interact with each other in double heterozygotes. This remarkable fact seems to indicate that the mechanisms by which these two genes produce their effects, at least so far as the inner ear is concerned, must be very similar. Under these circumstances a histological comparison of the cochlear changes seemed of interest. It was also hoped that a correlation of such changes with alterations in the electrical response of the cochlea might help to elucidate the still obscure problem of the origin of that phenomenon.

A histological examination of the labyrinth of adult shaker mice (29–444 days old) has been made by Meng (1933). He reports degeneration of Corti's organ from the age of 160 days onwards, followed later by degeneration of the spiral ganglion. No changes were observed in the stria vascularis. Meng's findings thus do not account for the deafness in younger shakers and do not include the early stages before the deafness develops.

It should be mentioned that there exists another kind of shaker mutation called shaker-2 (Dobrovolskaia-Zavadskaia, 1928) which is genetically different from both shaker-1 and waltzer; in its behaviour, it is practically indistinguishable from shaker-1. No data on the structure of its labyrinth have so far been published.

MATERIAL

The shaker animals used for this study were derivatives of a stock homozygous for the three linked genes for shaker-1, extreme dilution (an allelomorph of albinism, symbol c^d) and pink-eyed dilution (p) which had been built up for linkage studies by one of us (Grüneberg 1936). As normal controls we used throughout animals of the black agouti Pure Line Strong CBA (substrain Grüneberg, 38th–39th generation of brother-sister mating).

The hearing tests were of a rough kind. Mice with a normal sense of hearing respond by a twitching movement of their ears and to some extent of their whole bodies to a slight clicking sound produced by a pair of forceps. No such response can be elicited in the case of deaf animals.

The material is divided into two groups:

Group I. In this group, a histological examination only was made of the internal ears of one shaker and one normal animal at the following ages after birth: 9 and 24 hr., 4, 6 and 12 days. Tests of hearing were not carried out in any of these animals.

Group II. In this group, measurements of the electrical response of the cochlea with subsequent histological examination of the internal ear were carried out upon shaker and normal animals at the following ages: 38, 50, 117, 216, 221, 296 and 329 days. Hearing tests were carried out in every case.

TECHNIQUE

A. *Histological*

All material was fixed by intra vitam injection into the aorta of Witmaack's fluid, preceded by Ringer's fluid at 37° C. A series of stainless steel hypodermic needles graded to fit the aorta at varying stages of development were used, and in all early post-foetal material it was found necessary to introduce the needle under a low power of the dissecting microscope.

Thereafter the heads were further fixed in Witmaack's fluid followed by hardening in 4 % formaldehyde and embedded in celloidin.

Sections of the whole head were finally cut in the horizontal plane at 25μ and stained with Ehrlich's haemotoxylin and eosin.

B. *Electrical*

Exposure of the tympanum was carried out under urethane anaesthesia, injected subcutaneously. The animal was immobilized upon a cork slab in the dorsal position by means of pins through the extremities. A midline incision was then made from chin to sternum. The thyroid gland was removed and the carotid vessels ligated. The muscles and other deep tissues were then dissected down to the bulla and were widely separated by means of small metal retractors suitably shaped and controlled by screw mechanisms.

A small brass cannula was tied into the external auditory meatus and the bulla was then opened. The initial opening was made with a dental drill and was then enlarged by nibbling with small artery forceps. The round window lies quite superficially and was generally exposed quite easily. On a few occasions troublesome haemorrhage occurred from the stapedial artery in its upward course along the margin of the window.

For acoustic stimulation a pure tone beat-frequency oscillator was used, and the tones were led to the ear of the preparation via thick-walled rubber tubing.

The amplification system was of the conventional type with resistance-capacity coupling, a diffuse earthed electrode being located in the neck muscles and a grid electrode at the round window.

The most suitable form for this recording electrode was found to be a fine gauge platinum wire with a ball-point (conveniently produced in any small arc gap). The size of the ball was arranged to have a radius of curvature rather exceeding that of the round window membrane. Thus, after arranging the electrode in a manipulator at right angles to the window it was then possible to screw the ball down into firm contact with its bony margins, and without any danger of rupturing its delicate membrane. In this way a firm and uniform contact was obtained.

The amplified potentials were measured directly upon the face of a cathode-ray oscillograph and their amplitude thereupon matched by sinusoidal potentials of identical frequency derived from the beat frequency oscillator and introduced into the input circuit via a calibrated potentiometer.

Measurement of the matching potentials introduced in this way was made by means of an A.C. voltmeter connected across the outer terminals of the potentiometer. (For further details of the sound generating and amplifying equipment used, reference may be made to an earlier publication (Hallpike and Rawdon-Smith 1934 *a*).)

As in an earlier series of similar measurements in the cat, these were again made for the maximum voltages obtainable at the following four frequencies: 256, 512, 1024 and 2048 ~.

In addition, a comparison, expressed in decibels, was made of the sound intensities at the threshold of the electrical response as seen upon the tube face, with the audibility threshold of a normal hearing human subject. For this purpose, the sound tube was branched to bring the ears of observer and preparation into symmetry with respect to the sound source.

In making the measurements some trouble was encountered in maintaining a good base-line at high amplification. This was probably connected with the mode of fixing the head whereby small transmitted respiratory movements at the electrode contact point could not wholly be eliminated.

This difficulty is unlikely to have impaired materially the measurements of maximum response, except in the case of readings given as absent. Here a recognizable response might obviously have been obtainable with a better base-line.

In the case of the threshold measurements, however, the difficulty was more serious.

Immediately following the completion of the electrical investigation intra-vital fixation of the preparation was carried out in the manner already described.

RESULTS

Group I, 9 hr.–12 days

At birth the differentiation of Corti's organ in the mammal is still incomplete. Details of the course of this post-foetal development are given by Retzius who describes it as being complete in about 14 days in the rabbit and cat.

All of the present material was found to be well fixed, and has shown that development in the mouse appears to be complete by the 12th day. No differences could be observed in the structure of the scala media complex of normal and shaker animals at any stage up to 12 days.

Figures 1 and 2 show the organ of Corti in normal and shaker mice at the 12-day stage.

Group II, 38–329 *days*

A. *Normal.*

All the animals gave a normal hearing reaction (p. 156).

All of the material examined was found to be well fixed, and no significant variations in structure were encountered. Photographs of the cochlear structures found at 50 days appear in figures 3–5, and may be taken as typical of all of the normal material examined.

B. *Shakers.*

Hearing reactions were absent in every case.

All of the material was found to be well fixed. Pathological changes confined to the scale media complex were present in every case* and were studied chiefly in sections lying about the mid-modiolar plane. In such sections three half-turns of the cochlear spiral appear and will be referred to as the apical, middle and basal half-turns.

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* In a preliminary series of animals used for the development of the histological technique one adult shaker (age unknown) did not show visible changes in Corti's organ. It is thus possible that the pathological processes in that organ are not of invariable occurrence, although no similar exception was otherwise encountered in the present work.

The following components of the cochlear duct were considered in detail:

(1) Corti's organ.
(2) The spiral ganglion.
(3) The stria vascularis.
(4) Reissner's membrane.
(5) The tectorial membrane.

(1) *Corti's organ.* Three stages of degeneration were recognizable. In the first, degeneration had occurred of some but not all of the outer hair cells with disappearance of the nuclei of the affected cells.

In the second stage, there was complete degeneration of all the outer hair cells with disappearance of their nuclei, while in the third stage Corti's rods had disappeared and Corti's organ was reduced to a small and compact hillock of undifferentiated cells.

(2) *The spiral ganglion.* Changes in density of the ganglionic tissue were well marked. Three stages of such variation were recognized. In the first of these stages there was a just perceptible thinning of the ganglion cells. In the second, this thinning was of a grosser character, and the total bulk of the remaining ganglion cells was judged to approximate very roughly to 50 % of the available space in Rosenthal's canal. In the third stage an extreme degree of thinning had occurred and the ganglionic tissue was now reduced to a few scattered remnants.

These judgements were made by inspection of low-power views of the affected cochlea and by immediate comparison with similar views in normal members of the same age groups. Methods of greater precision such as counting of the ganglion cells were not employed.

Changes in the individual ganglion cells were much less readily discernible and not at all until the 50-day stage. Normally the ganglionic mass shows a characteristic dovetailed pattern which is achieved by a moulding of the cells at the expense of their cytoplasm. The nuclei are invariably well rounded with well defined chromatin granules and nuclear membrane. The first visible change occurred in some but not all of the shakers of the 50-day age group and consisted of a shrinkage of the cytoplasm resulting in a loss of the dovetailed appearance of the cells and a more open appearance of the cell mass.

In addition, certain nuclear changes could be made out. The nuclei; while retaining their staining characteristics, showed irregularities of shape, angular and kidney forms being the commonest (figures 6 and 7). Similar changes were present in all subsequent age groups and showed a generally progressive character with age.

In later stages both nucleus and the remnants of the cytoplasm tended to lose their staining reactions and disintegrate, resulting in irregular ghost forms.

The character of these changes and its bearing upon the further question of their mode of origin will be discussed later in greater detail.

(3) *The stria vascularis.* This was examined for changes in histological structure and secondly for changes in bulk.

(α) *Changes in histological structure.* Details of the normal structure of the stria are of interest inasmuch as thereon is based much that has been inferred concerning its function as the secretory source of the endolymph. In the mouse it consists of a layer of cells some 25 μ in thickness, of which the superficial cells are closely set with their nuclei near the surface and with irregular tapering processes of cytoplasm on their deep aspect. Deeper still there are other polyhedral epithelial cells haphazardly arranged with similar irregular processes of cytoplasm. The cell outlines are typically very ill-defined and irregular and this is in part responsible for the very characteristic fibrillated appearance of the stria in the mouse. Scattered about in the cells are numerous yellowish brown pigment granules, some of which in the immature stria are described by Kolmer (1927) as giving staining reactions for glycogen.

A further important feature of the stria is its rich vascular supply. In a radial section of the stria numerous small vessels are seen, generally in cross section, of which some are typical thin-walled capillaries. Others, however, have thicker walls of more arteriolar type and are described by Eckert-Möbius (1926) as pre-capillaries.

Whether the endolymph makes its way between the surface epithelial cells of the stria or whether, as Shambaugh (1932) has suggested, it escapes lower down between the cells lining the external spiral sulcus has not yet been established.

The structural features of the stria vascularis as they appear in the normal mouse are shown in figure 5.

Inspection of a high power view of the normal stria shows that a percentage of the total area, which obviously exceeds 50 and is probably about 70, is occupied by the fibrillated cytoplasmic material described.

In the present shaker material a type of degeneration could be distinguished, consisting of a loss of this cytoplasm with a relative increase of the nuclei and vascular spaces.

Three stages of this degeneration were recognizable. In the first, the cytoplasmic content of the stria showed a just appreciable reduction. In the second, the reduction of the cytoplasm was more extreme, and its total area was judged to approximate very roughly to 50 % of the total area of the stria. In the third stage, the cytoplasm was practically non-existent and the stria consisted of a few nuclei and vascular spaces tightly enclosed by the nuclei of the superficial and basal cell layers.

As in the case of the density changes in the spiral ganglion, the changes in the cytoplasmic content of the stria vascularis were assessed by inspection and no exact measurements were made.

(β) *Changes in bulk.* These were computed by measuring the area of the stria in a radial section of the basal half-turn of the cochlea. This was done by making a pencil tracing of the outline of the stria from a camera lucida projection at known magnification upon paper of a convenient and known mass per unit area. These tracings were then cut out with scissors and weighed. After correction for magnification, the resulting areas were expressed in μ^2.

Owing to variations in the pitch of the cochlear spiral towards the apex, similar measurements of the stria for the middle and apical half turns were found to be untrustworthy.

(4) *and* (5). *Reissner's and the tectorial membranes.* No changes were observed in these structures in any of the present material.

The vestibular apparatus. No changes were apparent in the cristae of the semicircular canals or of the maculae. Scarpa's ganglion appeared normal in density and cell structure.

MODE OF PRESENTATION OF RESULTS IN GROUP II

The degree and distribution of the degenerative changes encountered in Corti's organ, the spiral ganglion and in the stria vascularis have been presented in tabular form. The numbers 1, 2, 3, which appear in the tables, refer to the 1st, 2nd and 3rd degrees of degeneration described, and the column headings, A, M, B, denote the apical, middle and basal half-turns of the cochlea. The area of the stria in the basal half-turn is expressed in μ^2. The maximum voltages of the cochlear response are expressed in microvolts (peak) under the frequency headings 256, 512, 1024 and 2048 ~.

The threshold of the cochlear response is expressed in decibels above the normal human subjective threshold, the comparison being made in the manner already described (p. 157). No precise measurements were made above 60 db.; and thresholds above this level are tabulated as >60 db.

TABLE 1. NORMAL

No. of animal	Age	Corti's organ			Spiral ganglion			Stria vascularis			Area of stria	Cochlear response Maximum voltage				Cochlear response Threshold			
		A	M	B	A	M	B	A	M	B	μ^2	256	512	1024	2048	256	512	1024	2048
331	38	—	—	—	—	—	—	—	—	—	6885	156	190	190	67	60	44	> 60	> 60
349	50	—	—	—	—	—	—	—	—	—	7049	28	78	112	34	34	52	56	54
352	50	—	—	—	—	—	—	—	—	—	7052	224	56	112	123	46	52	56	54
345	117	—	—	—	—	—	—	—	—	—	7704	*	*	44	134	42	42	52	56
346	117	—	—	—	—	—	—	—	—	—	5754	201	134	252	224	54	54	54	60
347	117	—	—	—	—	—	—	—	—	—	6147	78	112	112	112	44	54	60	48
348	117	—	—	—	—	—	—	—	—	—	7540	90	123	168	156	40	54	42	42
355	216	—	—	—	—	—	—	—	—	—	5901	56	179	212	179	48	58	50	52
356	216	—	—	—	—	—	—	—	—	—	6147	224	201	224	212	54	44	48	48
358	216	—	—	—	—	—	—	—	—	—	6475	145	190	212	196	42	54	46	54
359	221	—	—	—	—	—	—	—	—	—	7459	56	56	112	56	46	48	58	54

* Brisk response observed but no measurements made.

TABLE 2. SHAKERS

No. of animal	Age	Corti's organ			Spiral ganglion			Stria vascularis			Area of stria	Cochlear response Maximum voltage				Cochlear response Threshold			
		A	M	B	A	M	B	A	M	B	μ^2	256	512	1024	2048	256	512	1024	2048
332	38	0	0	1	0	0	0	1	2	1	4016	—	—	—	—	—	—	—	—
333	38	0	0	2	0	0	0	1	1	0	5000	—	—	—	—	—	—	—	—
353	50	1	1	1	0	0	0	1	2	1	4754	56	3	—	—	60	> 60	—	—
354	50	1	1	1	0	0	0	2	2	1	4918	22	0·7	—	—	> 60	> 60	—	—
336	117	1	1	3	0	0	3	2	2	1	4426	—	14	—	—	—	> 60	—	—
337	117	1	2	2	0	0	0	3	2	1	3934	224	112	14	—	38	> 60	> 60	—
338	117	1	2	2	0	0	0	2	2	1	3770	14	7	—	—	> 60	> 60	—	—
339	117	2	1	2	0	0	0	2	2	2	4262	—	22	—	—	—	> 60	—	—
334	216	1	3	3	0	1	2	3	2	1	3934	—	—	—	—	—	—	—	—
335	216	2	2	3	0	0	2	3	2	2	3770	45	6	1	—	56	> 60	> 60	—
343	216	2	2	3	3	2	2	3	2	2	3770	—	—	—	—	—	—	—	—
344	221	2	2	3	0	0	3	3	2	1	4262	56	22	—	—	> 60	> 60	—	—
314	296	2	2	3	2	3	3	2	2	2	3196	—	—	—	—	—	—	—	—
315	296	2	2	2	0	2	2	3	2	2	3268	—	—	—	—	—	—	—	—
310	329	1	2	3	2	2	3	3	3	1	3772	—	—	—	—	—	—	—	—

Photomicrographs of the cochlea of the shaker at 38, 117 and 216 days appear in figures 8–16.

The following conclusions are derived from the tables.

TABLE 1. NORMAL

No structural abnormalities were encountered in Corti's organ, the spiral ganglion, or the stria vascularis.

The area of the stria vascularis. This varied between 5754 and 7704 μ^2 with an average value of 6737 μ^2. Variation with age was not apparent.

Maximum voltage of the cochlear response. It is of interest to compare the range and average value of the results with similar measurements upon the cat already published (Ashcroft, Hallpike and Rawdon-Smith 1937):

	Range		Average	
	Cat	Mouse	Cat	Mouse
256 ~	180–940	28–224	500	126
512 ~	230–900	56–201	511	132
1024 ~	300–1100	44–252	697	159
2048 ~	100–1000	34–212	518	136

This comparison shows that the form of the frequency-maximum voltage curve is much the same in both species.

Both have a peak at 1024 ~ which is slightly less pronounced in the mouse.

The voltage values in the mouse were found to be approximately 20–25 % of those obtained in the cat.

Threshold of cochlear response. Davis and his collaborators (Stevens, Davis and Lurie 1936) have made considerable use of the so-called threshold of the cochlear response under suitable conditions of electrical recording, and have found that it corresponds closely with the normal subjective human threshold. In a later review (Stevens and Davis 1938, p. 313) they recognize that this "threshold" is essentially a function of the resolving power of the electrical system in use. Assigning to this a probable value of 1 μV, it may thus be said that the 1 μV level of the response corresponds in the cat to the minimum audibility curve in man.

In the mouse the threshold, according to the present measurements, lies some 40–55 db. higher. In part at least this must be due to the loss of resolution of the electrical system due to the technical conditions already described (pp. 157–8).

The figures may be summarized as follows (animal no. 331 being excluded):

cyc./sec.	Range	Average
256	34–60	47
512	42–58	55
1024	42–60	54
2048	42–60	52

Threshold values showed no recognizable correspondence with maximum voltage responses.

TABLE 2. SHAKERS

Degenerative changes were present in every case in Corti's organ, the spiral ganglion, and the stria vascularis.

Corti's organ. There is a progressive degeneration with age. On the whole the basal half-turn is affected earliest and most severely.

Spiral ganglion. No gross changes are visible until the 117-day stage although, as described, finer changes in the nuclei can be distinguished in some members of the 50-day group. Thereafter the changes are progressive with age with some accentuation in the basal half-turn.

Stria vascularis. Degenerative changes are present in every case, and are progressive with age. The figures indicate an increase of the degenerative process towards the apex.

The area of the stria vascularis. This varied between 3196 and $5000\mu^2$, with an average value of $4077\mu^2$. There is no definite diminution with age.

Maximum voltage of cochlear response. The response was absent or grossly reduced in all cases. Certain correlations are of interest:

(1) There is in general a falling off of the maximum voltage of response with age, corresponding, that is to say, with the degeneration of the stria vascularis and of Corti's organ. Striking exceptions are the first two animals, 332 and 333, in which an absent response occurred with relatively slight morphological changes.

(2) The higher frequencies show a selective reduction. There was no response at 2048 ~ throughout the series and a small response at 1024 ~ in a single instance.

This is correlated with the accentuation of the degenerative changes of Corti's organ in the basal half-turn of the cochlea.

Comment upon these correlations will be made later.

The threshold of the cochlear response. This is grossly reduced throughout, but shows in general a quite close correspondence with the results of the maximum voltage measurements.

DISCUSSION

The questions arising from the foregoing data may be considered under two headings—pathological and electrophysiological.

(1) *Pathological*

In the first place the present findings correspond closely with those described by van Lennep in the case of the Japanese waltzing mouse inasmuch as in both species the morbid anatomical process in the organ of hearing is enacted after birth.

In the absence, therefore, of any morphological abnormalities at birth it is impossible to regard the condition as congenital.

In one particular the present findings differ from those of van Lennep in that no changes are recognizable in either the stria vascularis or in the cells of Corti's organ 12 days after birth, at which stage the process of development appears to be complete.

It thus follows that the degenerative process in the shaker is initiated in a fully differentiated organ.

The similarity in the cochlear pathology thus revealed in these two mutants leads to a partial understanding of the remarkable interaction between the genes for waltzing and shaking which has been shown to occur in the double heterozygotes, and it is regretted that it has been impossible to proceed with an investigation which had been planned of the cochlear structure in these animals.

Adopting the familiar view point of Streeter, which he applies to a number of other inherited malformations, we may now say that what is inherited in the shaker mouse is a localized organ weakness. The embryological history of such an organ would depend, it seems, upon the extent of this weakness and upon the nature and time of incidence of various unfavourable circumstances of environment. Thus a given combination of these factors may bring about an early arrest of development resulting in a congenital anatomical defect. At the other end of the scale appear various degenerative processes of the retina and different parts of the central nervous system which occur later in life. Granted in the present material the action of some unknown factor of environment in precipitating the process of degeneration, the establishment of its time relations does appear to be of interest in indicating in a very general way the direction towards which further search for such a factor might usefully be directed.

In other words, the initiation of the degeneration is concerned with one or more of the mechanical and biochemical changes which normally occur at or soon after the time of birth.

No comparison between deafness in the shaker-1 mouse and inherited deafness in man is possible on account of the scantiness of the data at present available on the developmental course of the cochlear changes in the latter. Nevertheless, the numerous histological examinations of the labyrinth in later life made by Scheibe (1891), Goerke (1906) and others clearly establish the fact that the disorder is an affection of the scala media with gross abnormalities of the stria vascularis and of Corti's organ, findings which also apply closely to the inherited deafness of white bull terriers (Fraser 1924) and white cats (Alexander and Tandler 1905). Thus it seems highly probable that the disorder in these species is of a type similar to that now disclosed in the shaker mouse, although there may well prove to be differences in the time of incidence and severity of the morbid anatomical changes.

Information of some importance appears to be provided by a consideration of the details of the pathological changes now described in the shaker mouse.

The changes first appear together in Corti's organ and in the stria vascularis. Those in the ganglion cells follow. It may be noted at this point that Meng (1933), in the only account at present available of the cochlear structure of the shaker, found changes in Corti's organ only and describes the stria vascularis as normal. In our own material the abnormalities of the stria are beyond question and an inspection of Meng's published photomicrographs makes it probable that his failure to demonstrate the changes in question may be attributed to unavoidable deficiencies of technique.

The parallel association in our material of the changes in the stria and Corti's organ is important as an illustration of the functional relationship between these structures. From the absence of capillaries in Corti's organ and the gland-like structure of the stria it has been inferred for some time that the metabolic processes of the former are carried on through the medium of the endolymph, a fluid which is thought to be secreted by the latter. This view has gained important support from Guild's demonstration (1927) of a flow of endolymph from the scala media to the saccus endolymphaticus via the saccule, and is clearly supported by the finding in the present material that the changes in Corti's organ and in the stria vascularis are so closely coincident.

It seems reasonable to infer from the comparatively delayed onset of the changes in the cells of the spiral ganglion that these are secondary to the changes in the peripheral fibres in Corti's organ, an important conclusion since it disposes of an alternative possibility that the changes in the cochlear duct and in the ganglion are all part of a single degenerative process affecting in equal measure all parts of the peripheral organ of hearing.

A question which naturally arises at this point is why degeneration of the ganglion cells should in any case be expected to follow degeneration of Corti's organ, and to this it would seem that a likely answer can be provided from what is now known concerning the behaviour of the ganglion cells following interference with their central processes.

Witmaack (1911), Kaida (1931), and, more recently, Hallpike and Rawdon-Smith (1934 *b*) have shown that contrary to Waller's law section of the VIIIth nerve at the internal auditory meatus is followed by severe degeneration of the peripheral cochlear neurones, although in accordance with the law the cells of Scarpa's ganglion, after showing transient changes, persist unaltered. Similar findings have been reported by Crowe (1929) and by Gray and de Kleijn (1932) as a result of pressure upon the VIIIth nerve by tumours; and by Mellanby (1938) *following* compression and stretching of the nerve by the masses of new bone which occur in vitamin A deprivation. This atypical behaviour of the spiral ganglion cells is best explained on the supposition that it reflects an extreme sensitivity of the cells to interference with their central axons; the transient changes which are classically described as occurring in cell bodies following section of their axons being consequently of unusual severity and therefore irreversible. Upon this basis it would thus appear reasonable to expect a similar degeneration following injury or disease of their peripheral processes in Corti's organ, as revealed in the present material.

Collateral evidence in favour of this conclusion is provided by the fact that degenerative changes of the ganglion cells follow the destruction of Corti's organ by acoustic trauma, changes which are regarded by Yoshü (1909) and Hoessli (1912) as being secondary to the injury of the peripheral organ and its associated nerve fibres.

With regard finally to the details of the cell changes it is true that the cytoplasmic dissolution and the changes of shape of the nucleus are not in accordance with the classical picture of secondary degeneration of nerve cells, namely peripheral displacement of the nucleus and chromatin material. Nevertheless it seems unjustifiable, as Mellanby (1934) has pointed out, to anticipate that such findings derived from artificial lesions of the neurones would be generally applicable to pathological conditions in which the nature and mode of application of the injurious agent is likely to be very different.

The absence of visible changes in the vascular supply of the internal ear and in particular of the stria vascularis contra-indicate the likelihood

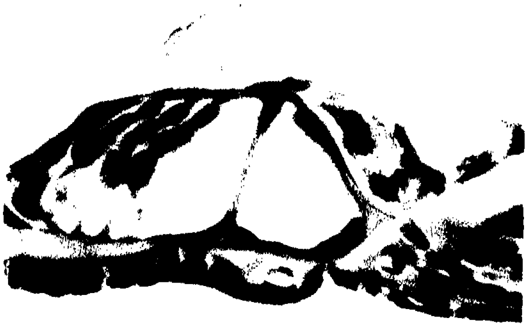

Figure 1

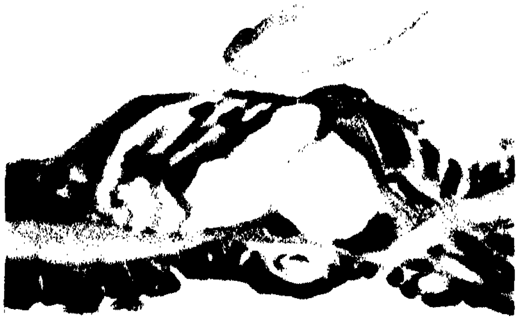

Figure 2

FIGURES 1 and 2. Organ of Corti of normal mouse (1) and shaker (2). × 525. Age 12 days. Development complete. Structure normal.

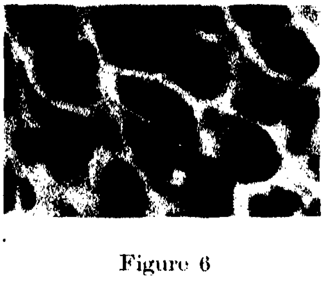

Figure 6

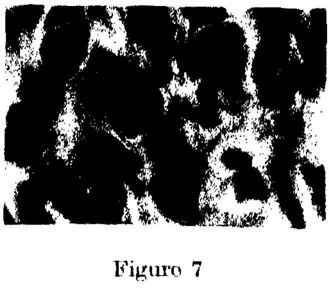

Figure 7

FIGURES 6 and 7. Spiral ganglion of normal mouse (6) and shaker (7). × 740. Age 117 days. Showing the character of the degenerative changes in the latter.

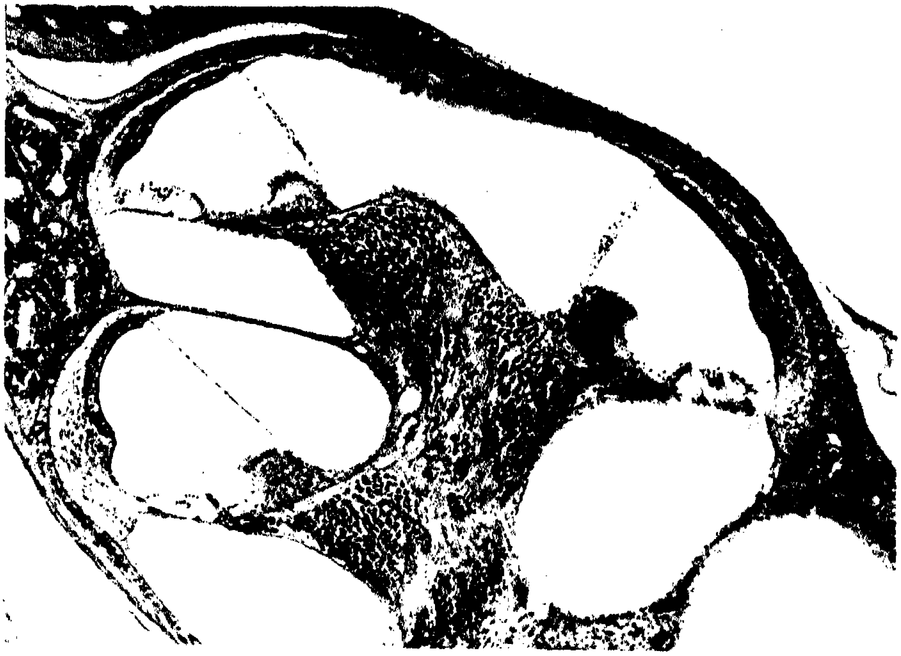

Figure 3

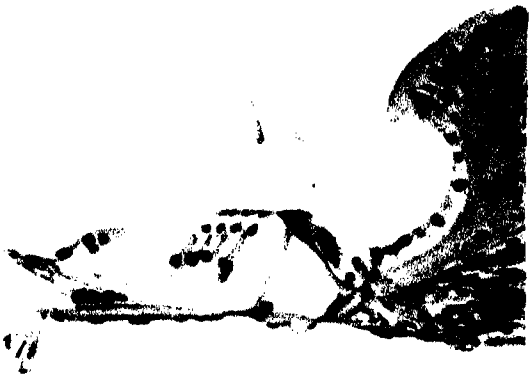

Figure 4

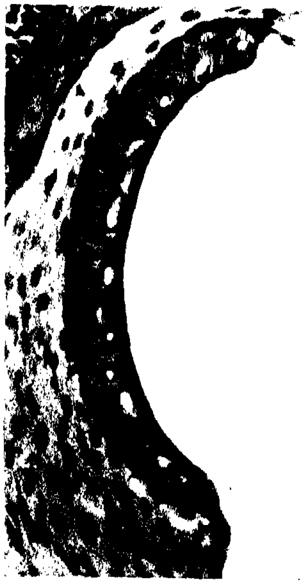

Figure 5

FIGURES 3–5. Cochlear structure of normal mouse. Age 50 days. 3. Cochlea. × 85. 4. Organ of Corti. × 300. Basal half-turn. 5. Stria vascularis. × 285. Basal half-turn.

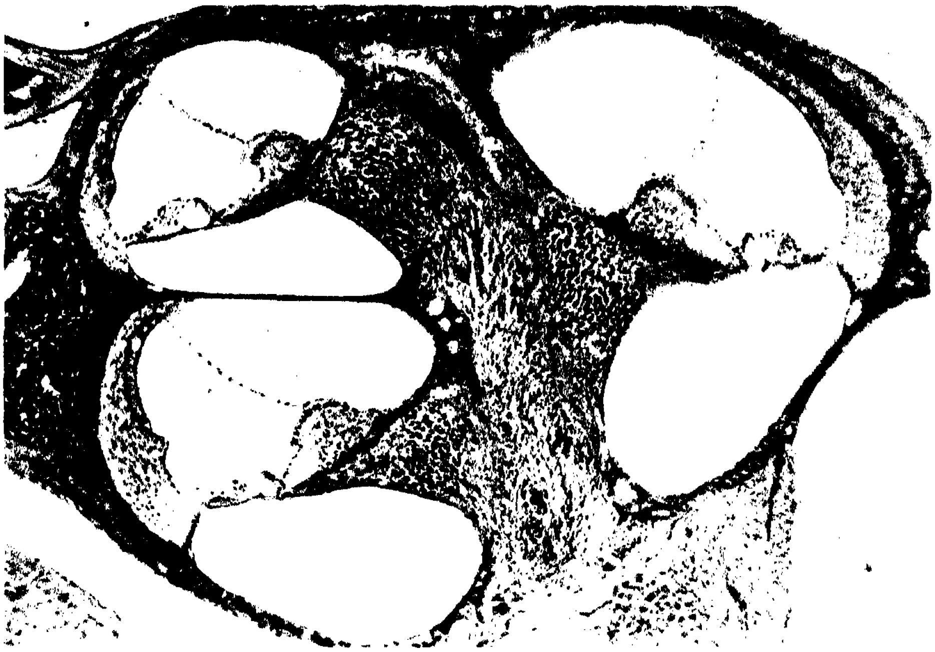

Figure 8

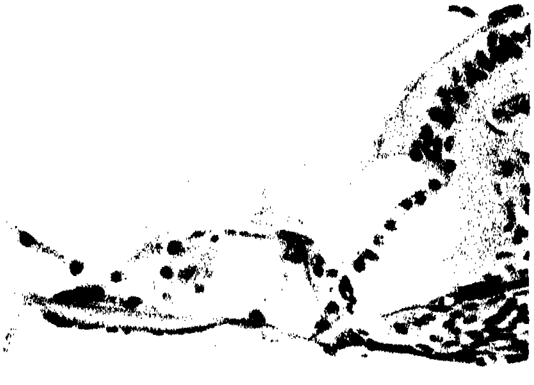

Figure 9

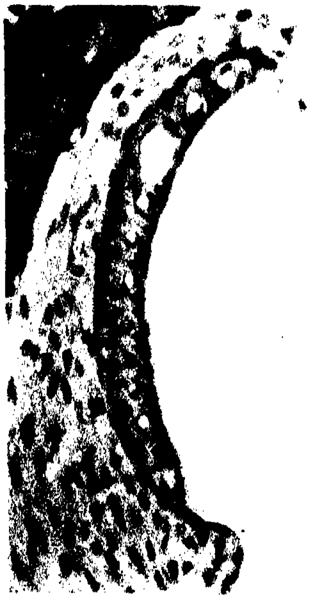

Figure 10

FIGURES 8-10. Cochlear structure of shaker mouse. No. 332. Age 38 days.

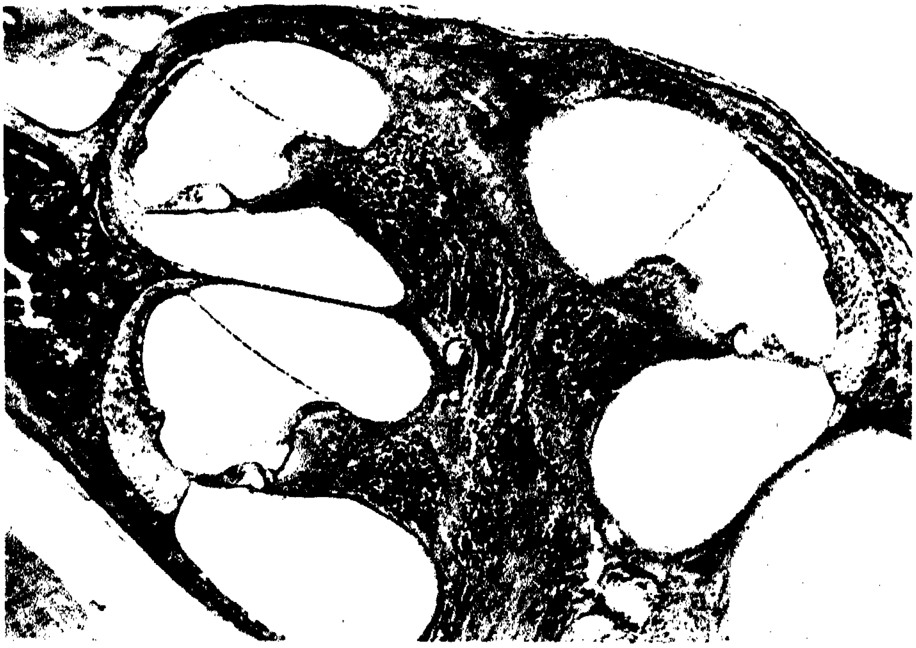

Figure 11

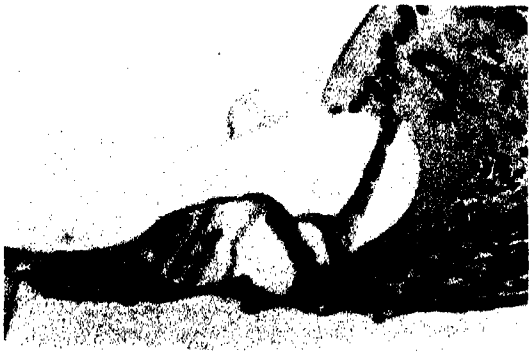

Figure 12

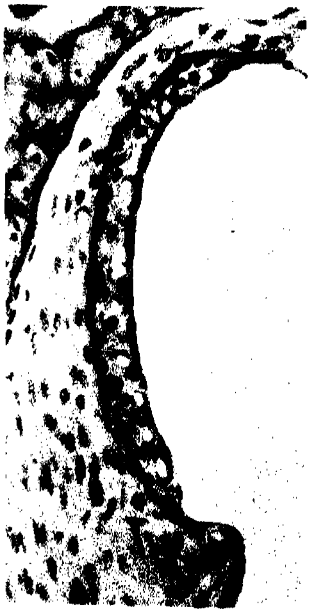

Figure 13

FIGURES 11–13. Cochlear structure of shaker mouse, No. 337. Age 117 days.

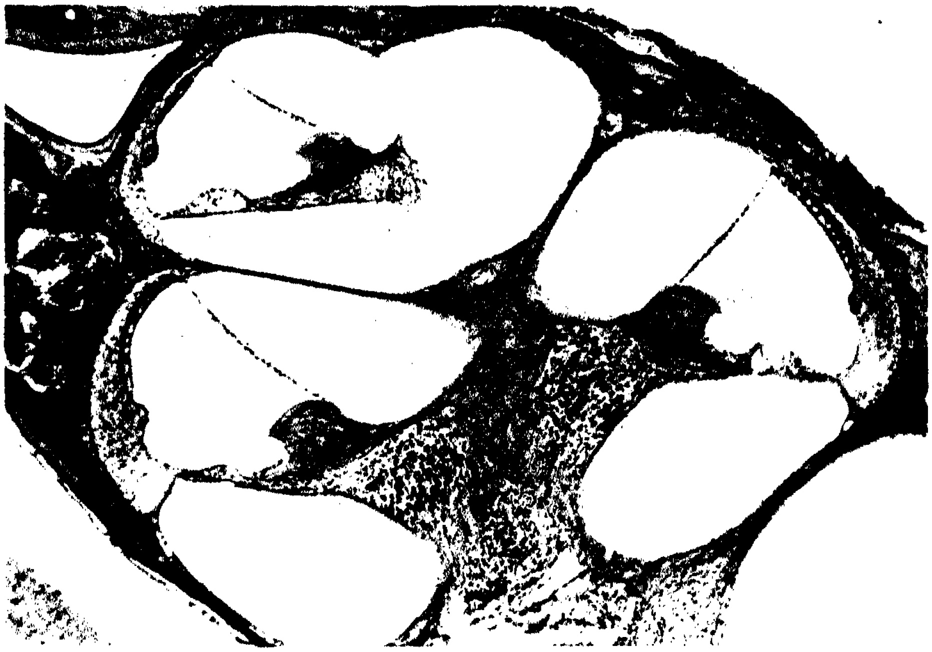

Figure 14

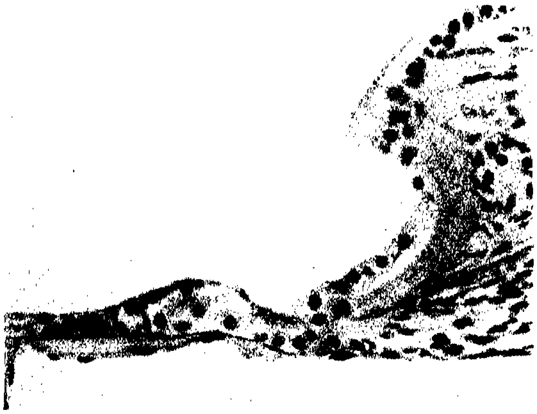

Figure 15

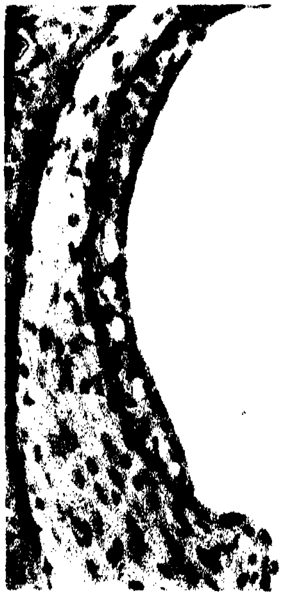

Figure 16

FIGURES 14–16. Cochlear structure of shaker mouse, No. 343. Age 216 days.

that the degenerative processes described are primarily of vascular origin.

Similarly, the demonstration of normal conditions in the subarachnoid space and in the internal ear around the opening of the cochlear aqueduct render improbable the invasion of the labyrinth by some meningogenic toxin by way of this aqueduct. The absence of demonstrable changes in the vestibular end organs makes it likely that the characteristic shaking movements are of central origin. This would accord well with the demonstration by Zimmermann (1935) of changes in the corpus striatum of the shaker.

(2) *Electrophysiological*

No decisive solution to the problem of origin of the electrical response to sound of the mammalian cochlea has yet been provided.

Two hypotheses at present hold the field, namely the "Hair-cell theory" of Davis and his collaborators (1934) and the "Membrane theory" of Hallpike and Rawdon-Smith (1934).

The evidence for and against these two hypotheses has been reviewed by Stevens and Davis, Hallpike, and by Hallpike and Rawdon-Smith, and will not be reproduced here.

According to the "Hair-cell" theory the response arises in the manner of a piezo-electric effect from the hair cells of Corti's organ, while Hallpike and Rawdon-Smith have supposed that it arises from the movements of some polarized membrane within the cochlea. They suggested in particular the membrane of Reissner separating the endolymph canal of the cochlea from the perilymph scala.

According to the original terms of the membrane theory the supposed polarization of Reissner's membrane was thought to depend upon a difference in the ionic concentrations of the endolymph and the perilymph, a difference which was in turn made dependent upon the known difference in the modes of origin of these two fluids.

This view now appears to be contra-indicated by the recent experiments of Walzl (1939) who found that the electric response was comparatively insensitive to changes in the ionic composition of the perilymph.

In the face of this evidence, however, it still seems clear that its acceptance would not exclude the membrane hypothesis inasmuch as the polarization mechanism might well be integral in the membrane itself.

As Retzius has described, the membrane is composed of a double layer of cells, ectodermal on its endolymphatic and mesodermal on its perilymphatic aspects. Such a membrane might well display an electrical asymmetry such as described by Du Bois Reymond and others in the case of intact frog's skin, and one moreover which would be relatively independent of the ionic composition of the surrounding fluids.

Whether the polarization of Reissner's membrane were dependent upon the preservation of a particular ionic composition of the endolymph or upon the electrical asymmetry of the membrane itself, it seems clear that the maintenance would in either case be required of an unimpaired secretion of the endolymph. In the first case, obviously, in order to maintain the required composition of the endolymph and in the second case in order to keep the membrane alive, since, as now seems likely, the vital processes of this structure as of Corti's organ are equally subserved through the medium of the endolymph. Thus the membrane hypothesis must in either case continue to depend upon the functional integrity of the stria vascularis.

The present investigation has shown a progressive loss with age of the cochlear response. Since, however, the concomitant degenerations of both stria vascularis and of Corti's organ pursue strictly parallel courses, it follows that the results are indecisive as to the relative merits of the membrane and hair-cell hypotheses.

Certain anomalous results were obtained in two animals at the earliest stage of 38 days. In these, absence of electrical response was correlated with relatively trifling changes in Corti's organ, results which were strikingly reminiscent of a number of earlier findings in the cat (Hallpike and Rawdon-Smith 1934 *b*; Ashcroft, Hallpike and Rawdon-Smith 1937) in which a morphologically intact cochlear duct was found associated with absence or gross reduction of the electrical response.

On morphological grounds alone such a result would seem contrary to both hair-cell and membrane hypotheses. At any rate the former, since it is likely that functional changes in the highly differentiated structure of Corti's organ would be more prone to reflection in recognizable histological changes than in the case of the less delicate and differentiated cell structure of Reissner's membrane and of the stria vascularis. On such grounds the findings in question have been considered as being particularly opposed to the hair-cell hypothesis, a view which can still with reason be maintained.

In a more general sense, however, the findings certainly emphasize the somewhat indirect and at times histologically inscrutable nature of the relationship between microscopic structure and the electrical response, and it seems conceivable that the temporary absence of the latter at the 38-day stage of our shaker material, that is to say at a time coincident with the onset of the degenerative changes, is indicative of some acute but transient disturbance of cochlear metabolism which occurs at this critical period in the absence of any recognizable morphological changes. However, pending the experimental confirmation of these findings further speculation would clearly appear to be unjustifiable.

One other finding of considerable interest has been the correlation between the distribution within the cochlea of the degeneration of Corti's organ and the frequency distribution of the changes in the electrical response.

Thus there is a well-marked selective loss of the response at the higher frequencies associated with an accentuation of the degenerative changes in the hair cells of the basal half turn of the cochlea. This appears to be in striking accordance with results obtained by Stevens, Davis and Lurie (1936) in which localized lesions of Corti's organ were found to result in losses of the electrical response having a frequency pattern conforming with the classical resonance hypothesis, results which were considered to lend particularly strong support to the hair-cell theory. An alternative interpretation must, however, be considered. As described by Davis *et al.* (1934) and since confirmed (Hallpike 1938), the polarity of the electrical response at the round window with reference to an indifferent electrode farther removed from the cochlea depends upon the phase of the sound stimulus, a positive sound pressure giving a negative electrical response and vice versa. In addition, Davis has described a neural component of the electrical response recordable from the round window which is particularly marked at low frequencies but negligible above 800 ∼. These findings have been confirmed by Hawkins and Rawdon-Smith (1939), and our own observations show that the phase relationship of this neural component to the cochlear response is usually such as to reduce its amplitude. Thus the abolition of this neural element would lead to a relative enhancement of the cochlear response at low frequencies and the end result of a progressive reduction of the cochlear response would then be its selective retention at these frequencies. Such an abolition of the neural component in the present material would necessarily result from the degenerative changes found in the cells of the spiral ganglion at the 50-day and subsequent stages; in addition the changes in Corti's organ would, of course, eliminate the neural response at its source. In this way it would therefore seem possible to explain the present finding of a selective retention of apical hair cells and low frequency electrical response without postulating a localized origin of the cochlear response from the hair cells.

CONCLUSIONS

Histological examination of the post-foetal development of the internal ear in normal and shaker mice was carried out at periods between 9 hr. and 12 days after birth.

The normal process of post-foetal development appeared to pursue an identical course in both varieties and to be completed in 12 days.

Tests of hearing, measurements of the electrical response of the cochlea and histological examination of the internal ear were carried out in normal and shaker mice at ages between 38 and 329 days. In all the shaker material deafness was found to be correlated with pathological changes confined to the scala media complex and gross reductions of the electrical response. The changes first appear in the stria vascularis and Corti's organ and are progressive with age. Those in the spiral ganglion appear later.

The degenerative process is thus shown to be initiated after birth in a fully differentiated organ, and comparison is made with similar results obtained by van Lennep in the Japanese waltzing mouse.

The parallel courses of the loss of the electrical response and of the degenerative changes in the stria vascularis and in Corti's organ render the results indecisive with regard to the relative merits of the "membrane" and "hair-cell" theories of origin of the response.

A selective loss of the response was found to occur at high frequencies and is correlated with some accentuation of the degenerative changes in Corti's organ at the base of the cochlea. The resemblance of this to similar results obtained by Stevens, Davis and Lurie in the guinea pig under different experimental conditions is discussed, and the results are not considered to constitute a decisive contradiction of the membrane hypothesis.

No morphological abnormalities were found in the vestibular apparatus of the internal ear. It is therefore supposed that the abnormal head movements are of extra-labyrinthine origin.

The authors are much indebted to Professor A. de Kleijn for kindly providing a copy of van Lennep's thesis, together with an English translation of its conclusions.

Acknowledgments are also due to the Medical Research Council for the provision of an assistant to one of us (C.S.H.). The expenses of the histological work were borne in part by a grant from the Gamble Research Fund of the Royal Society of Medicine.

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The reversible formation of starch from glucose-1-phosphate catalysed by potato phosphorylase

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An enzyme (phosphorylase), which catalyses the reversible conversion of starch and inorganic phosphate to glucose-1-phosphate, has been found to occur in a number of higher plants.

A method is described for obtaining from potato tubers highly active preparations of phosphorylase which are free from certain enzymes which catalyse alternative reactions.

The reversibility of the transformations: starch + inorganic phosphate $\rightleftharpoons$ glucose-1-phosphate, is shown by the fact that the reaction proceeds in either direction until the ratio of inorganic orthophosphate/glucose-1-phosphate attains a value which is not significantly altered by wide variations in the concentrations of the reactants or the enzyme.

The equilibrium value of this ratio is not detectably affected by alterations in the concentration of starch but varies considerably with alterations in the concentration of hydrogen ion. This is shown to be due to the effects upon the extents of dissociation of the inorganic and esterified phosphate, the di-valent ions alone determining the equilibrium; thus the ratio

$$(\mathrm{HPO_4})^{=}/(\mathrm{C_6H_{11}O_5\,.\,O\,.\,PO_3})^{=}$$

remained approximately constant at a value of 2·2 when the pH value was varied from 5 to 7.

When glucose-1-phosphate is added to the enzyme there is an initial lag in the liberation of phosphate and the formation of starch. This induction phase is abolished, and the reaction velocity greatly increased, by small additions of starch or maltose.

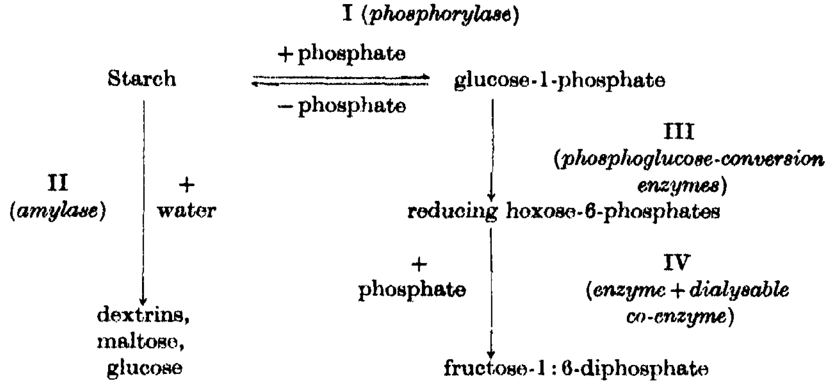
The preparation on a large scale of glucose-1-phosphate (in the form of the crystalline potassium salt) by allowing crude potato juice to act in starch is described.

Observations on the polysaccharide formed from pure glucose-1-phosphate under the action of purified phosphorylase indicate that it resembles closely the so-called amylo-amylose fraction of natural potato starch.

The mechanism of this reversible interconversion and its bearing on problems of carbohydrate metabolism in the plant are considered.

INTRODUCTION

It was shown in Part I of this series (Hanes 1940*a*) that extracts from ungerminated peas contain an enzymic system which, in the presence of inorganic phosphate, catalyses the formation of hexosephosphates from starch or various saccharides of the starch-maltose series. The first stage in this transformation, catalysed by the enzyme phosphorylase, consists in the conversion of starch to glucose-1-phosphate and this was found to be a reversible reaction. Both starch and glucose-1-phosphate, however, take part in alternative reactions, catalysed by separate enzymes, which are indicated in the following schema:


I (*phosphorylase*)
+ phosphate
Starch
glucose-1-phosphate
− phosphate
III
(*phosphoglucose-conversion enzymes*)
II
(*amylase*)
+
water
reducing hexose-6-phosphates
+
phosphate
IV
(*enzyme + dialysable co-enzyme*)
dextrins, maltose, glucose
fructose-1:6-diphosphate

This series of reactions appears to be closely related to those which occur when glycogen is acted upon by the enzyme systems of yeast and various animal tissues; of particular interest is the fact that the conversion of glycogen into glucose-1-phosphate has also been shown to be a reversible reaction. This was demonstrated by Schäffner and Specht (1938) and Kiessling (1939*a*, *b*) using yeast phosphorylase; by Cori, Schmidt and Cori (1939) with muscle phosphorylase; and by Ostern and Holmes (1939), Cori, Cori and Schmidt (1939), and Ostern, Herbert and Holmes (1939) with preparations from liver.

The object of the experiments here presented was to examine in detail the reversible reaction I of the above schema. For this purpose it was desirable that preparations of phosphorylase should be available which were free from the enzymes which catalyse reactions II and III. A method for obtaining such preparations from extracts from peas was devised, and preliminary observations on these have already been reported (Hanes 1940*a*).

In the meantime, however, the enzyme phosphorylase has been found to be present in many higher plants, crude aqueous extracts or the juice pressed from different organs (leaves, roots, fruits and tubers) of a number of species exhibiting varying degrees of activity (cf. Hanes 1940*b*). The potato is the most favourable source of the enzyme so far encountered. The crude juice pressed from the tubers, after grinding, contains an active phosphorylase but is devoid of phosphoglucose-conversion activity. By a relatively simple procedure the phosphorylase has been purified considerably, and the weak amylase activity exhibited by the crude juice has been eliminated. These purified preparations of potato phosphorylase have been used in most of the ensuing experiments.

METHODS

Analytical technique

As in the previous investigation, the enzyme and other components of a digest were brought separately to 25° C before mixing, and the reaction was followed at this temperature. All solutions and digests were kept saturated with toluene.

The first sample for analysis was withdrawn 0·5–0·7 min. after initiating the reaction and others at appropriate times to define the progress of the reaction. In addition initial values were determined by separate analyses on the various digest components; these were in close agreement with initial values obtained by extrapolating the progress curves to zero time.

The analyses consisted mainly in determinations of inorganic orthophosphate (*free-P*) and phosphate in the form of glucose-1-phosphate (*ester-P*), the latter corresponding to the increase in *free-P* which resulted when samples were heated for 7 min. at 100° in the presence of *N* perchloric acid.

The digest samples were normally of 1 ml. and these were delivered into 5 ml. 6 % trichloracetic acid to arrest the reaction and precipitate protein. After standing 5–10 min. the precipitate was filtered off and, as soon as possible, a suitable volume of the filtrate for the determination of *free-P* (usually 1 ml.) was transferred to a 25 ml. volumetric flask and neutralized with dilute ammonia, about 15 ml. water and a drop 0·1 % phenolphthalein being added. For the determination of *free-* + *ester-P* a second 1 ml. portion of the filtrate, with 4 ml. water and 1 ml. 6 N perchloric acid, was heated for 7 min. in a test-tube suspended in boiling water; after cooling, the contents were transferred, with several washings, to a 25 ml. flask.

The determinations of phosphate were made by an improved method elaborated by Allen (1940) in this laboratory; as before, the colour intensity was measured by an absolute photometer using a deep red filter. This procedure had the advantage, in addition to increased reproducibility, that the necessary period of contact of the sample with the acid-molybdate-reducer mixture was considerably shortened since the colour developed to a steady maximum intensity in 5 min. (as compared with 20 min. in the method used previously).

In practice, analyses were done on batches of six to eight samples; in the case of samples for the determination of *free-P*, the average period of contact with 5 % trichloracetic acid before neutralization was about 12 min. and the period in contact with 0·77 N perchloric acid of the phosphate reagent about 10 min. at the time the reading was made. It was found by separate experiment that a slight hydrolysis of glucose-1-phosphate occurred under these conditions amounting to 0·4 %. All values for *free-P* and *ester-P* have been subjected to this barely significant correction, values of *ester-P*, derived from (*free-* + *ester-P*) − *free-P*, being increased by 0·4 % and values of *free-P* being corrected by substracting this absolute amount.

All values have been given in terms of mg. P per 10 ml. digest; similarly, the composition of digests are given as for 10 ml. (final volume) digest although in practice the volumes ranged from 5 ml. to 8·5 l.

The glucose-1-phosphate used had been prepared by the action of pea phosphorylase on starch according to the method described earlier (Hanes 1940*a*). It was in the form of potassium salt which had been twice re-crystallized and of which the composition was in close agreement with the formula $C_6H_{11}O_5.O.PO_3.K_2.2H_2O$. It was completely hydrolysed to glucose and *free-P* under the conditions described for the determination of *ester-P*. Quantities of the ester specified below will refer to this crystalline (hydrated) salt containing 8·31 % *ester-P*.

Inorganic phosphate when added to digests was in the form of buffers prepared from potassium dihydrogen phosphate and sodium hydroxide.

In many experiments maleate buffers, prepared as described by Temple (1929), were used. It may be mentioned that when the reaction: glucose-1-phosphate → starch + *free-P*, is in progress the solution becomes more alkaline, and when the reverse reaction occurs it becomes more acid. This effect, resulting from the increased dissociation of the esterified phosphate groups as compared with free phosphate, has been observed in other esterification processes (cf. Harden 1932).

Preparation of potato phosphorylase

Small King Edward potatoes in batches of four to six (about 200 g.) were crushed in a large mortar and, after the addition of toluol, were pounded to a fine pulp. The juice was pressed out through canvas in a screw press, 100–120 ml. being obtained from 200 g. tissue.

This crude juice was used as enzyme in only a few experiments but, as will be shown below, it can be used without further treatment for large-scale preparations of glucose-1-phosphate.

Dialysis of the juice against distilled water caused complete inactivation of the phosphorylase, the result apparently of the irreversible flocculation of protein which occurred.

Some purification could be achieved by treating the juice at its natural acidity (pH 5·8–6·0) with calcium phosphate, kaolin or alumina C_{γ}, followed by filtration or centrifuging. Much inactive material was removed by adsorption with relatively small losses of phosphorylase, but the preparations still contained amylase.

The bulk of the experiments have been carried out with phosphorylase preparations which were devoid of amylase activity and contained either no *free-P* or a mere trace. These were obtained by the following method.

To 600–1000 ml. crude juice was added an equal volume of saturated ammonium sulphate. The resulting precipitate could not be removed quantitatively on the centrifuge but was separated by gravity filtration at 0° C, which required 6–12 hr. The precipitate was taken up in water (about one-half the original volume of juice); saturated ammonium sulphate was added until the density reached 1·085 (i.e. about 0·3 saturation) and the copious precipitate which formed was removed on the centrifuge and discarded. The fraction rich in phosphorylase was then precipitated by increasing the ammonium sulphate in the supernatant liquor to about 0·5 saturation (density 1·145) and was removed on the centrifuge. It was now dissolved in water of about one-third the original volume and the fractionation was repeated, i.e. a small amount of material precipitated by 0·3 saturated ammonium sulphate was removed and discarded. The main bulk of the enzyme was precipitated from the supernatant solution with 0·5 saturated ammonium sulphate and recovered by centrifuging. This moist precipitate was dissolved in water and diluted to give a convenient concentration of enzyme (usually one-third to one-half the volume of original juice); the pH was adjusted to 5·9–6·1 by the addition of dilute ammonia and the solution was stored under toluene at 0° C.

Such solutions of purified potato phosphorylase are relatively stable, losses in activity of the order of 10 % occurring during 14–20 days at 0° C. They contain 1·5–3 % ammonium sulphate in addition to 0·3–0·5 % of material derived from the potato juice. This highly active fraction, apparently protein in nature, represents 3–5 % of the dry matter present in the crude juice and possesses 70–80 % of the original phosphorylase activity.

EXPERIMENTS WITH CRUDE AND WITH PARTIALLY PURIFIED POTATO PHOSPHORYLASE

The consideration of a few experiments with crude potato juice and with preparations partially purified by adsorptive treatments serve to illustrate a number of features of the system which will be re-examined in later experiments with purified preparations.

TABLE 1. EXPERIMENTS WITH CRUDE POTATO JUICE

Digest 1. 10 ml. containing 6·25 ml. juice with 50 mg. soluble starch, 1·25 ml. M/2 phosphate of pH 8·5. pH of digest: initial value 7·4, final 7·1.

Digest 2. As digest 1 except that phosphate at 6·4 was added. pH: initial 6·3, final 6·0.

Digest 3. 10 ml. containing 7·5 ml. juice with 50 mg. glucose-1-phosphate. Final pH: 6·1.

Results as mg. P per 10 ml.

Digest		Time (min.)					
		0	10	20	40	100	160
1	*free-P*	21·5	19·8	18·9	18·3	17·6	17·7
	free- + ester-P	21·7	—	—	—	21·5	21·6
	Ratio*	**0·99**	—	—	—	**0·82**	**0·82**
2	*free-P*	21·6	19·5	19·1	18·9	18·8	18·8
	free- + ester-P	21·6	—	—	—	21·5	21·6
	Ratio*	**1·00**	—	—	—	**0·875**	**0·87**
3	*free-P*	1·35	3·80	5·09	5·18	—	5·18
	free- + ester-P	6·02	(6·02)	6·05	6·00	—	6·02
	Ratio*	**0·22**	**0·63**	**0·84**	**0·86**	—	**0·86**
	Iodine colour	Yellow	Blue-green	Blue	Blue-violet		Blue-violet

* Ratio: *free-P*/*free- + ester-P*.

The observations in table 1 demonstrate the presence of an active phosphorylase in potato juice. In digests 1 and 2, which consisted of crude juice with additions of soluble starch and inorganic phosphate, there occurred a progressive decrease in *free-P*, with the formation of an acid-labile ester. As will be shown later this ester is glucose-1-phosphate. It is to be noted that more glucose-1-phosphate was formed ultimately in digest 1 of which the final pH was 7·1, than in digest 2 at pH 6·0.

The reverse reaction is illustrated in digest 3 to which glucose-1-phosphate equivalent to 4·16 mg. *ester-P* per 10 ml. digest was added. The *free-P* increased from 1·35 mg. initially (representing inorganic phosphate present in the crude juice) to 5·18 mg., with a corresponding decrease in *ester-P*. Accompanying this rapid liberation of *free-P*, the formation of polysaccharide was evident when samples were tested with iodine. The iodine coloration did not remain blue but soon developed a purple tinge; on standing for prolonged periods, e.g. 24–48 hr., the iodine coloration in such digests became pale violet or rose, and ultimately disappeared.

A 10 ml. sample of digest 3 removed after 35 min. was treated with 10 ml. 8 % trichloracetic acid; after filtering off the precipitate, which contained considerable blue-staining polysaccharide, 3 vol. of 95 % alcohol was added to the filtrate. The resultant precipitate was removed on the centrifuge and, after washing with alcohol, was dissolved in a little water. With iodine it gave a blue-violet coloration; the iodine-colouring property was rapidly destroyed when α-malt- or salivary amylase was allowed to act on portions of the solution. Preliminary experiments of this type showed clearly that a starch-like substance was formed by the action of phosphorylase on glucose-1-phosphate. The preparation of large samples of the polysaccharide was not attempted using crude juice, since there was evidence that it was being slowly degraded by the action of the amylase present in such preparations.

Attention may be drawn finally to the drifts in the ratio *free-P*/*free-* + *ester-P*. In digest 2 the reaction: starch + *free-P* → glucose-1-phosphate, proceeded until this ratio attained a steady value of 0·87; in digest 3, of which the final pH was approximately the same, the reverse reaction proceeded until the ratio reached the same value. As will become clear later, the only factor which has been found so far to alter appreciably the equilibrium value of the ratio is the concentration of hydrogen ions; a preliminary indication of this effect is to be seen in the observations on digests 1 and 2.

Table 2 gives the results of experiments with juice which had been treated with calcium phosphate at pH 5·8. To 100 ml. juice was added 20 ml. of a suspension containing 0·2 g. tri-calcium phosphate in a highly adsorptive form; the mixture was stirred for 10 min. and then centrifuged. The supernatant solution was used as enzyme, digests 4–8 being carried out shortly after preparing the enzyme when it was still pale yellow in colour, and digests 9–11 on the following day when it had become dark brown.

In one respect the observations on this particular preparation are unique and for this reason they have been selected for presentation. Thus in digests 5, 7 and 8 there occurred a significant decrease in the value *free-* + *ester-P*. This has not been observed in any other of the numerous experiments with different potato preparations (including enzymes prepared by this same method). Since, however, the effect may have been due to the

TABLE 2. EXPERIMENTS WITH CALCIUM PHOSPHATE-TREATED JUICE

Results as mg. P per 10 ml.

			Time (min.)					
Digest	**Additions**		0	5	11	25	50	90
Digests 4–8. 10 ml. containing 6·7 ml. freshly prepared juice, with other additions as stated. Final pH: 6·1–6·3.								
4	**Water only**	*free-P*	1·40	—	—	—	1·45	1·48
		free-+ester-P	1·49	—	—	—	1·54	1·55
5	**22·2 mg. Gl.-1-P***	*free-P*	1·41	1·73	2·24	2·77	2·80	2·78
		free-+ester-P	3·38	3·40	3·40	3·41	3·24	3·24
		Ratio†	**0·42**	**0·51**	**0·66**	**0·81**	**0·865**	**0·86**
6	**22·2 mg. Gl.-1-P* + 1 ml. 0·056 M phosphate**	*free-P*	3·15	3·44	—	4·18	4·35	—
		free-+ester-P	5·09	5·10	—	5·00	5·06	—
		Ratio†	**0·62**	**0·675**	—	**0·835**	**0·86**	—
7	**22·2 mg. Gl.-1-P* + 22·2 mg. soluble starch**	*free-P*	1·43	2·26	2·82	2·80	2·76	—
		free-+ester-P	3·41	—	3·37	3·32	3·25	—
		Ratio†	**0·42**	—	**0·835**	**0·845**	**0·85**	—
8	**44·4 mg. Gl.-1-P***	*free-P*	1·43	1·89	2·56	4·07	4·18	3·96
		free-+ester-P	5·20	—	5·21	5·22	4·81	4·56
		Ratio†	**0·275**	—	**0·49**	**0·78**	**0·87**	**0·87**
			Time (min.)					
			0	5·5	13	25	63	90
Digests 9–11. 10 ml. containing 6·7 ml. of the same preparation after storage for 24 hr. at 0° C.								
9	**22·2 mg. Gl.-1-P***	*free-P*	1·55	1·70	2·17	2·76	2·92	2·96
		free-+ester-P	3·40	3·41	3·44	3·44	3·36	3·38
		Ratio†	**0·455**	**0·50**	**0·60**	**0·805**	**0·870**	**0·875**
10	**As No. 9 but enzyme pre-treated with 2·5 mg. sodium hydrosulphite**	*free-P*	1·53	1·72	2·17	2·76	2·96	—
		free-+ester-P	3·44	(3·45)	3·46	3·44	3·43	—
		Ratio†	**0·445**	**0·50**	**0·625**	**0·80**	**0·865**	—
11	**22·2 mg. Gl.-1-P* + 2 mg. soluble starch**	*free-P*	1·56	2·34	2·84	2·92	2·96	—
		free-+ester-P	3·46	(3·46)	3·47	3·44	3·42	—
		Ratio†	**0·450**	**0·675**	**0·82**	**0·85**	**0·865**	—

* Gl.-1-P = glucose-1-phosphate.

† Ratio: *free-P*/*free-+ester-P*.

presence of a trace of phosphoglucose-conversion activity it seemed desirable to record this exceptional feature. The effect was absent after the enzyme had been stored for 24 hr. (digest 9) and was not regenerated by treatment of the enzyme with sodium hydrosulphite which reduced the brown pigments (formed from oxidation) to pale yellow (digest 10). This latter experiment was suggested by the observations of Gill and Lehman (1939) which indicated that the phosphoglucose-conversion system of muscle is reversibly inhibited by various oxidants.

So far as the phosphorylase activity was concerned the data are typical of those obtained with other partially purified preparations. These normally contained a trace of *ester-P*, and on incubation a slight increase in *free-P* occurred, as in digest 4.

In digests 5 and 9, with added glucose-1-phosphate, the reaction: glucose-1-phosphate → starch + *free-P*, proceeded until the ratio *free-P*/*free-* + *ester-P* attained values of about 0·87, but it will be noticed that there was an initial phase during which the reaction proceeded slowly. This induction phase is also observed when crude juice or purified preparations are used.

Digests 7 and 11 show the effect of additions of soluble starch, together with glucose-1-phosphate, upon the reaction. In each case the initial velocity was greatly increased but the final value of the ratio was not appreciably altered.

The observations of digests 6 and 8 indicate that with the addition of inorganic phosphate, and with double the amount of glucose-1-phosphate, respectively, the final values of the ratio were the same.

So far as could be judged from qualitative observations on the iodine coloration, the amounts of starch formed in these different digests appeared to be at least roughly proportional to the amounts of *free-P* liberated; thus the final blue-violet colour given by digest 8 was much more intense than in digest 5 and this in turn was perceptibly deeper than digest 6 (in which the final extent of the conversion of glucose-1-phosphate was repressed by the addition of *free-P*).

EXPERIMENTS WITH PURIFIED POTATO PHOSPHORYLASE

Five different preparations of potato phosphorylase, purified by fractional precipitation with ammonium sulphate as described above were used in the ensuing experiments. Certain general observations may be mentioned at the outset.

These solutions of purified phosphorylase were devoid of both phosphoglucose-conversion and amylase activity; further, the *free-P* and the small amount of *ester-P* present in crude juice had been almost completely eliminated during the purification.

When glucose-1-phosphate was added to the enzyme the first sign of starch formation was a green coloration when samples were tested with iodine; this was clearly the resultant of the yellow-staining flocculence of protein which formed when the enzyme alone was tested, and the blue-staining of traces of synthesized starch. Provided sufficient ester had been added, the iodine colour became blue-green and then deep blue as the reaction progressed, and heavy floccules of the blue-black starch-iodine complex were formed. In contrast to digests with crude preparations, the iodine colour remained blue even during prolonged periods of incubation (e.g. 10 days).

When relatively high concentrations of glucose-1-phosphate were added it was observed consistently that a large proportion of the synthetic starch was deposited in the form of definite grains during the later stages of the reaction. These were approximately spherical in shape and, in different experiments, ranged from 4 to 10 μ in diameter. The formation of these starch grains first became evident from a turbidity in the clear reaction mixture; later, there appeared on the bottom of the vessel a white sediment which consisted of the microscopic grains, frequently in adherent clusters. The individual granules, which stained blue-black with iodine, appeared sometimes to be surrounded by flocculent material which stained yellow but this was not always the case.

It is not yet clear whether the deposition of the starch in the form of granules is part of the enzymic process or whether it is due to a physical process of molecular aggregation. As will be seen later, the synthetic polysaccharide exhibits to a marked degree the property of retrograding from solution. It deserves mention that Meyer (1895) reported the deposition of "artificial starch grains" from concentrated solutions of starch.

THE EFFECT OF ADDITIONS OF STARCH AND VARIOUS SUGARS ON THE INDUCTION PHASE

With all preparations of potato phosphorylase an initial induction phase in the formation of starch and *free-P* from glucose-1-phosphate has been observed. This was illustrated by the data in table 2 which demonstrated the additional fact that the presence of soluble starch increased greatly the initial reaction velocity.

This phenomenon was examined in greater detail in a series of digests

TABLE 3. EXPERIMENTS WITH PURIFIED POTATO PHOSPHORYLASE SHOWING THE EFFECT OF ADDITIONS OF STARCH WITH GLUCOSE-1-PHOSPHATE

Digest 12 (control). 10 ml. containing 2·86 ml. enzyme, N/42 maleate buffer, but without glucose-1-phosphate.

Digests 13–19*. 10 ml. containing 2·86 ml. enzyme, 29 mg. glucose-1-phosphate, N/42 maleate buffer, additions of soluble starch as stated. Final pH values: 6·3–6·5.

Results as mg. P per 10 ml.

			Time (min.)								
Digest	mg. starch		0	2	3	9	20	30	44	50	65
12 (control)	0	*free-P*	0·04	—	—	—	—	—	—	—	0·04
		free-+ester-P	0·04	—	—	—	—	—	—	—	0·05
13	0	*free-P*	0·055	—	—	0·25	0·63	1·115	1·86	—	2·06
		free-+ester-P	2·45	—	—	(2·46)	2·48	2·46	2·47	—	2·45
		Ratio†	**0·02**	—	—	**0·101**	**0·250**	**0·453**	**0·755**	—	**0·838**
17	2·9	*free-P*	0·05	—	0·53	1·36	1·92	—	—	2·06	—
		free-+ester-P	2·48	—	(2·47)	2·47	(2·47)	—	—	2·47	—
		Ratio†	**0·02**	—	**0·215**	**0·551**	**0·781**	—	—	**0·837**	—
19	28·6	*free-P*	0·07	0·61	—	1·745	2·04	—	—	2·07	—
		free-+ester-P	2·47	2·45	—	2·46	2·45	—	—	2·46	—
		Ratio†	**0·03**	**0·250**	—	**0·709**	**0·833**	—	—	**0·841**	—

* Detailed analyses are given for three typical digests of this series. The results for the whole series are plotted in figure 1.

† Ratio: *free-P*/*free-+ester-P*.

(12–19) with purified enzyme. Full analytical data are given in table 3 for typical digests of this series.

The control digest 12 contained only a trace of *free-P* and no *ester-P* and no changes occurred during incubation. In the remaining digests *free-P* was liberated at the expense of *ester-P*. As in all experiments with purified enzyme, the value *free-* + *ester-P* remained constant.

In figure 1 the values of the ratio *free-P*/*free-* + *ester-P* for the whole series of digests are plotted against reaction time. It is clear from these progress curves that the addition of soluble starch, even in very low concentration, increased the reaction velocity, and in higher concentration it had the effect of abolishing completely the induction phase. The relationship between the initial reaction velocity and the amount of added starch is shown in figure 2 A; maximum activation was attained with the addition of 10–12 mg. starch per 10 ml.

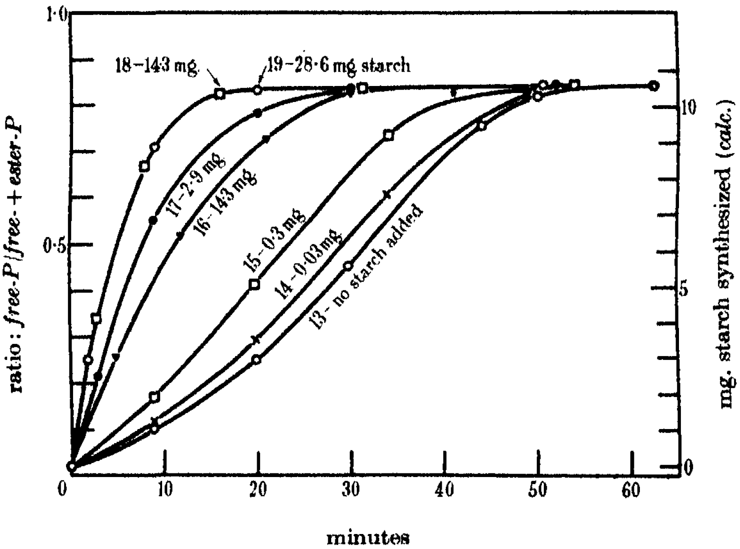


FIGURE 1. Digests 13–19 (described in table 3) showing the effects of additions of starch upon the conversion: glucose-1-phosphate→*free-P* + starch. Digest 13 had no starch added, digests 14–19 had the amounts indicated on each progress curve (expressed in mg. per 10 ml. digest).

These observations suggest that in digests without added starch the factor which conditions the progressive acceleration of the reaction during the induction phase is the accumulation of the starch synthesized from glucose-1-phosphate. A point of interest is that soluble starch added initially to the digest causes a much greater activation than the same quantity of starch formed by the reaction itself. Thus it will be seen from the right-hand scale of ordinates in figure 1 that in digest 13 after 20 min. 3 mg. starch had been formed from glucose-1-phosphate; the initial addition of a slightly smaller amount of soluble starch to digest 17 produced a 4–5 times greater increase in velocity than had occurred spontaneously in digest 13. There is little doubt that the much greater activating effect of soluble starch as compared with the enzymically-synthesized starch is due to a difference in the physical state in the two cases, the latter being present in a highly aggregated form.

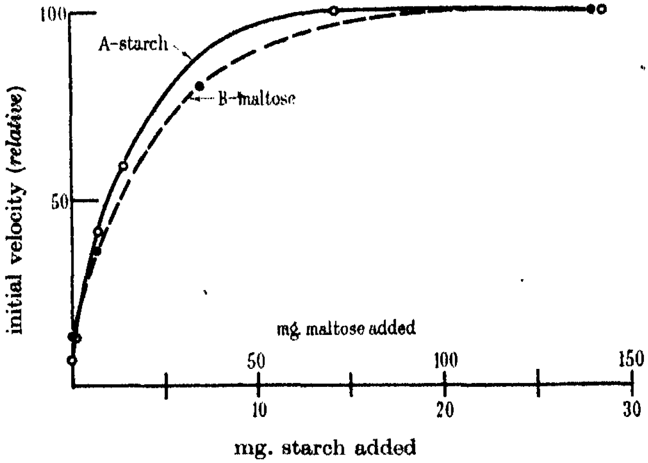


FIGURE 2. Comparison of the activating effects of starch (curve A) and maltose (curve B) upon the conversion: glucose-1-phosphate →*free-P* + starch. Initial velocity values (determined graphically) are expressed as percentages of the maximum value observed. (Different enzyme preparations were used in the two experiments.)

The interesting question arises as to whether the presence of starch is essential for the action of the phosphorylase. Thus it might be suggested that the enzyme preparation contained a trace of starch without which no synthesis of starch from glucose-1-phosphate would have occurred. According to this view, the amount of starch present in the enzyme would be estimated (by extrapolating the curve in figure 2 A to zero velocity) at about 0·03 mg. in the 2·9 ml. of enzyme added to each digest.

Further support for this view had been obtained in earlier experiments on the purification of the pea phosphorylase. Thus certain fractions from pea extracts (e.g. a fraction which remained in solution after precipitating the bulk of the enzyme with 0·5 saturated ammonium sulphate) showed considerable phosphorylase activity as judged by the rate at which they catalysed the reaction: starch + *free-P* → glucose-1-phosphate, but appeared at first to have no activity in catalysing the reverse reaction.* After periods varying from 0·5 to 2 hr., however, the liberation of *free-P* and synthesis of starch began, and proceeded at increasing velocity. This very pronounced induction phase could be completely eliminated by the addition of small amounts of soluble starch. It thus seemed likely that the prolongation of the induction period observed with these preparations was due to a more complete removal of traces of starch than was the case in the standard purification of the potato phosphorylase.

After this work was completed a parallel effect with (glycogen-) phosphorylase from muscle tissue was described by Cori and Cori (1939). With progressive purification by an adsorption method, this enzyme was reported to exhibit an increasingly marked induction phase in its action on glucose-1-phosphate; induction periods up to 24 hr. were observed in certain cases. The addition of small amounts of glycogen activated the enzyme, and abolished the induction phase. Cori and Cori concluded that traces of glycogen are required for the enzymic synthesis of glycogen from glucose-1-phosphate.

Before putting forward any suggestion as to the mechanism of this activation which is clearly common to the two types of phosphorylase, it should be stated that starch is not unique in producing the effect, at least in the case of the higher plant enzyme.

In table 4, for example, are shown the effects of additions of different sugars upon the reaction. Glucose, fructose, and cane sugar (digests 21, 22, 23) caused no increase in velocity but apparently inhibited the reaction slightly. Maltose, however, caused a considerable activation (digest 24) although the effect was smaller than that produced by the same amount of starch (digest 25). (It may be mentioned that the maltose used was a carefully recrystallized specimen.)

The addition of varying amounts of maltose was studied in a separate experiment (digests 26–29) the results of which have been plotted in figures 2 B and 3. It will be seen that maltose exerted an activating effect analogous to that of soluble starch but considerably larger additions of maltose were required to produce corresponding increases in reaction velocity.

In spite of the marked alterations in the velocity of the reaction resulting from additions of starch or maltose (figures 1, 2) the final extent of conversion of glucose-1-phosphate was not significantly affected. These experiments confirm the observations with crude preparations of phosphorylase.

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* Kiessling (1939*b*), having observed the somewhat similar behaviour of certain preparations, (glycogen-) phosphorylase from yeast and muscle, drew the conclusion that the phosphorylase must consist of two separate components which catalyse selectively the reactions proceeding in opposite directions. It is now unnecessary to point out the objections to this conception, since there is little doubt that the basis of the effect was similar to that described above.

A further aspect of the activating effects of starch and maltose upon

TABLE 4. EFFECT OF STARCH AND DIFFERENT SUGARS ON THE REACTION VELOCITY

Digests 20–24. 10 ml. containing 2·9 ml. purified potato phosphorylase, 29 mg. glucose-1-phosphate, N/42 maleate buffer; other additions were as stated; pH 6·1. Observations were made only on the liberation of *free-P*.

Results as mg. *free-P* per 10 ml. digest

Digest	Other additions	Time (min.)			
		0	10	30	60
20	None	0·07	0·18	0·44	0·85
21	20 mg. glucose	0·07	0·17	0·38	0·71
22	20 mg. fructose	0·07	0·16	0·34	0·75
23	20 mg. sucrose	0·07	0·16	0·38	0·85
24	20 mg. maltose	0·07	0·62	1·64	2·07
25	20 mg. starch	0·08	1·25	1·88	2·05

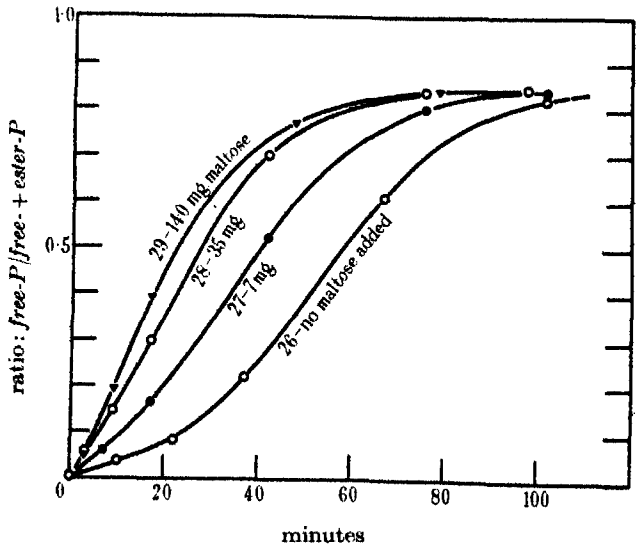

FIGURE 3. Digests 26–29 showing the effects of additions of maltose upon the conversion: glucose-1-phosphate → *free-P* + starch. The digests (10 ml.) contained 3 ml. purified phosphorylase, 29 mg. glucose-1-phosphate (initially) and N/42 maleate buffer (final pH 6·5). In addition the amounts of maltose indicated were added to digests 27, 28 and 29, none being added to digest 26.

the reaction deserves emphasis. Without additions of these substances the iodine coloration tends to be green during the induction phase, becoming blue in increasing intensity as the reaction proceeds; further, it has been found that almost the whole of the synthesized starch is precipitated with the protein when trichloracetic acid is added, the filtrates exhibiting only a faint violet hue. When the synthesis of starch is activated by initial additions of starch or maltose marked differences in behaviour were noticed.

Thus with small additions of starch (e.g. 1–2 mg. per 10 ml.) although a slight iodine colour is exhibited from the beginning, it was evident that the iodine colour given by the polysaccharide synthesized was at first purple and not blue; the bulk of it, moreover, was not precipitated by trichloracetic acid. In more advanced stages of the reaction the colour became blue and now trichloracetic acid filtrates contained only traces of iodine-staining material.

The effect of added maltose was even more striking. The polysaccharide which forms rapidly in the early reaction stages exhibited an unusual orange or brick-red iodine colour which later became crimson, violet, and ultimately blue. The products giving orange, red, and crimson colours were not precipitated by trichloracetic acid but the final blue-staining product is almost completely precipitated. With maltose concentrations up to 2–3 % (as in digests 26–29) the red coloration persisted longer the higher the maltose concentration. Other experiments with additions of maltose up to 8 % showed that there was some inhibition of the whole reaction but that the red-staining products which formed were not transformed into violet- or blue-staining polysaccharides even after prolonged reaction.

A detailed study of these effects may be expected to throw light on the mechanism by which the starch molecule is elaborated in this enzymic reaction. The aberrant iodine colours observed during the early reaction stages when starch and, more particularly, maltose are added suggest that there may be an accumulation under these conditions of certain intermediate compounds representing stages in the building-up of the complex aggregated system of starch molecules. A question of interest is whether the activating effect may not be due to the participation of certain groupings of the added saccharides (e.g. hydroxylated C atoms at position 4) in the early stages of the condensation reaction.

THE EFFECT OF HYDROGEN-ION CONCENTRATION UPON THE VELOCITY AND FINAL EQUILIBRIUM STATE

Three experiments will be considered, in the first of which observations were made on a series of digests (30–37) at pH values ranging from 5·2 to 7·6 to determine (*a*) the initial velocity and (*b*) the final extent of the conversion: glucose-1-phosphate → starch + *free-P*. In addition to glucose-1-phosphate a small amount of soluble starch was added to the digests to abolish the induction phase thus facilitating the determination of initial reaction velocity.

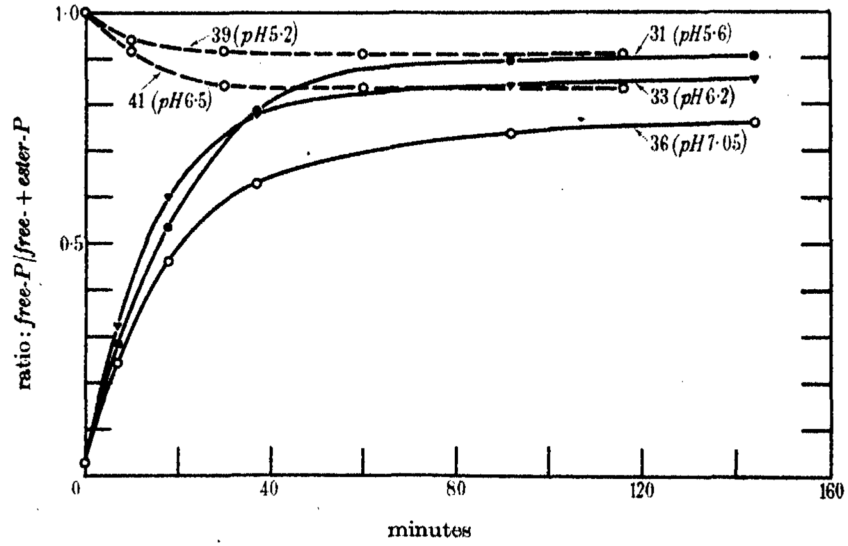

FIGURE 4. Digests 31, 33 and 36 (from the series described in table 5) illustrate the differential effects of varying concentrations of hydrogen ion upon the velocity and extent of the conversion: glucose-1-phosphate → *free-P* + starch. Digests 39 and 41 (from table 6) show the effects upon the reaction in the reverse direction.

The process curves in figure 4 for three digests of this series, illustrate the differential effects of hydrogen-ion concentration upon the velocity of the reaction and the final equilibrium attained.

The results for the whole series are given in table 5.

The initial velocity values exhibit a fairly sharp maximum in the region pH 5·9–6·1 (cf. figure 5 A, p. 193).

The values of the ratio *free-P*/*free-* + *ester-P* at equilibrium also show a dependence upon the concentration of hydrogen ions. Thus as the final pH values increased from 5·25 to 7·05, the values of the ratio progressively diminished, indicating lower final conversions of glucose-1-phosphate into polysaccharide at higher values of pH.

TABLE 5

Digests 30–37. 10 ml. containing 1·7 ml. purified phosphorylase solution, 33·3 mg. glucose-1-phosphate, 3·3 mg. soluble starch, N/36 maleate buffers. The initial and final pH values were as stated. Observations on *free-P* and *free-* + *ester-P* were continued for 144 min.; progress curves for digests 31, 33 and 36 are plotted in figure 4.

Digest	pH values		Initial velocity* mg. *free-P* liberated per 10 ml./min.	Ratio *free-P*/*free-* + *ester-P* at equilibrium
	Initial	150 min.		
30	5·2	5·25	0·056	**0·905**
31	5·5	5·6	0·100	**0·905**
32	5·75	5·85	0·115	**0·890**
33	6·05	6·2	0·121	**0·855**
34	6·4	6·5	0·116	**0·84**
35	6·6	6·75	0·104	**0·77**
36	6·9	7·05	0·097	**0·76**
37	7·6	7·6	0·069	—†

* The initial slope of the curve *free-P*/time, determined by the black thread method.

† Equilibrium not attained in the period under observation.

That this effect was not due to a failure of the system to reach equilibrium (e.g. due to differential rates of inactivation of the enzyme) was clearly demonstrated in the following experiments.

In digest 38 (40 ml. total volume, containing 15 ml. purified phosphorylase solution, 200 mg. glucose-1-phosphate, and N/48 maleate buffer; initial pH 6·1) the reaction was allowed to proceed to the equilibrium state. The ratio *free-P*/*free-* + *ester-P* after 100 min. was 0·85 and this was unchanged after 136 min.; the pH was now 6·35. A series of subdigests (38 *a*–*f*) was then set up. To 3 ml. portions of the original reaction mixture were added 1 ml. portions of different buffer solutions (acetate, veronal-acetate, and bicarbonate) which, as had been previously determined, resulted in a suitable range of pH values; in one case (38 *c*) 1 ml. water was added instead of buffer solution. Determinations of the ratio *free-P*/*free-* + *ester-P* were made on each of the subdigests after further reaction periods of 180 and 270 min. Except in the most alkaline digest (38 *f*) these showed that new equilibrium states corresponding with the adjusted pH values had been attained in 180 min. The final values of pH and ratio *free-P*/*free-* + *ester-P* were as follows:

Subdigest	38 *a*	38 *b*	38 *c*	38 *d*	38 *e*	38 *f*
Final pH	5·08	5·70	6·33	6·50	6·82	8·75
Ratio *free-P*/*free-* + *ester-P* at equilibrium	**0·92**	**0·88**	**0·85**	**0·80**	**0·775**	—

It was clear from these results that alterations in the hydrogen-ion concentration after equilibrium had been attained at pH 6·35 resulted in shifts to new equilibrium states; in 38 *c* which had remained at the original pH no change in the ratio had occurred; in 38 *a* and 38 *b*, which had been made more acid, further conversions of glucose-1-phosphate to starch and *free-P* had occurred, while in 38 *d* and 38 *e* under conditions of greater alkalinity conversions in the reverse sense had occurred.

Finally, data are given in table 6 showing the effects of hydrogen-ion concentration upon the formation of glucose-1-phosphate in digests to which starch and inorganic phosphate were added initially.

TABLE 6. THE FORMATION OF GLUCOSE-1-PHOSPHATE FROM STARCH AND INORGANIC PHOSPHATE AT DIFFERENT pH VALUES

Digests 39–43. 10 ml. containing 2·5 ml. purified potato phosphorylase, N/24 maleate buffers and varying amounts of inorganic phosphate and soluble starch. pH values, determined after the attainment of equilibrium, were as stated. Results for mg. *free-P*/10 ml. are given.

	Final	mg.		Time (min.)					
Digest	pH	starch		0	10	30	60	116	200
39	5·2	25	*free-P*	4·27	4·01	3·91	3·89	3·89	—
			Ratio*	**1·00**	**0·94**	**0·915**	**0·91**	**0·91**	—
40	6·0	25	*free-P*	4·22	3·98	3·74	3·63	3·65	—
			Ratio*	**1·00**	**0·94**	**0·885**	**0·86**	**0·865**	—
41	6·5	25	*free-P*	4·24	3·88	3·57	3·55	3·55	—
			Ratio*	**1·00**	**0·915**	**0·84**	**0·835**	**0·835**	—
42	6·0	40	*free-P*	19·8	18·9	17·9	17·6	17·5	17·3
			Ratio*	**1·00**	**0·955**	**0·905**	**0·89**	**0·885**	**0·875**
43	5·9	11	*free-P*	4·24	3·99	3·83	3·75	3·73	—
			Ratio*	**1·00**	**0·94**	**0·905**	**0·885**	**0·88**	—

* Ratio: *free-P*/*free-* + *ester-P*.

In digests 39, 40 and 41, which were comparable as regards the initial concentrations of starch and phosphate, the final extents of the esterification increased with the increase in pH values. The equilibria attained in these digests agreed approximately as regards the values of the ratio *free-P*/*free-* + *ester-P* with those observed at corresponding pH values in the preceding experiments in which the reverse reaction was followed.

The observations on digests 42 and 43 of which the pH values were similar to digest 40, show that, despite considerable differences in the initial concentrations of starch and phosphate, the same equilibrium state was finally reached. (It may be mentioned that when insufficient starch is added the true equilibrium state is not attained, the formation of glucose-

1-phosphate ceasing somewhat abruptly when the available starch has been transformed to the maximal extent.)

The two separate effects of hydrogen-ion concentration upon the system are shown in figure 5 A, B, in which are plotted the main observations from these three experiments.

Thus figure 5 A shows the pH-velocity relationship exhibited by potato phosphorylase when catalysing the reaction: glucose-1-phosphate → starch + inorganic phosphate.

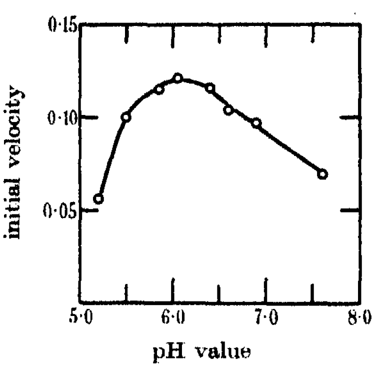

FIGURE 5 A

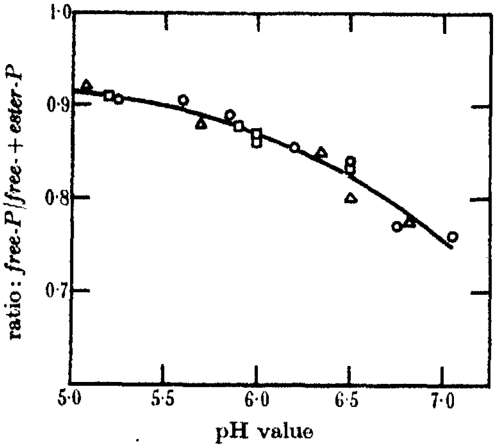

FIGURE 5 B

FIGURE 5 A. The pH initial velocity relationship observed for the conversion: glucose-1-phosphate → *free-P* + starch. The values were obtained from digests 30–37 (table 5).

FIGURE 5 B. The effect of hydrogen-ion concentration upon the equilibrium values of the ratio: *free-P/free-* + *ester-P*. Circles: digests 30–37 (table 5); triangles: digests 38 *a*–*e* (p. 191); squares: digests 39–43 (table 6).

Figure 5 B, on the other hand, shows the relationship between pH and the values of *free-P/free-P* + *ester-P* at equilibrium. The different sets of points plotted here, including values for the equilibrium state approached from both directions, are closely distributed about a single curve. There is thus no doubt as to the reversible character of the effect of hydrogen ion upon the equilibrium. It will be noted that under more acid conditions a larger proportion of the reactants exists in the form of starch and inorganic phosphate.

The full magnitude of this effect is not indicated directly by the values *free-P/free-* + *ester-P* adopted hitherto for defining states of equilibrium. In table 7 are set out the values of this ratio read off at different pH values from the smoothed curve in figure 5 B, and, for comparison, the corresponding values of the ratio *free-P/ester-P*. These it will be seen decreased from 10·8 to 3·1 as the pH value increased from 5·0 to 7·0.

TABLE 7. ANALYSIS OF THE EFFECT OF pH ON THE EQUILIBRIUM

Equilibrium at pH	5·0	5·5	6·0	6·5	7·0
Values of *free-P/free- + ester-P**	0·915	0·90	0·87	0·825	0·755
Equivalent values *free-P/ester-P*	10·8	9·0	6·7	4·7	3·1
$(HPO_4)^{=}$/total orthophosphate (calc.)	0·0149	0·046	0·132	0·326	0·602
$(C_6H_{11}.O_5.O.PO_3)^{=}$/total glucose-1-phosphate (calc.)	0·0695	0·191	0·422	0·701	0·881
$(HPO_4)^{=}/(C_6H_{11}O_5.O.PO_3)^{=}$ at equilibrium (calc.)†	2·3	2·2	2·1	2·2	2·1

* Values read off the curve in figure 5 B.

† Calculated from the ratio *free-P/ester-P*, e.g. at pH 5·0 this ratio was 10·8/1; the numerator was multiplied by 0·0149 and the denominator by 0·0695; hence the ratio $(HPO_4)^{=}/C_6H_{11}O_5.O.PO_3)^{=}$ was 0·1604/0·0695 = 2·31.

It seemed probable that the considerable variation in this ratio with changing pH was due primarily to effects upon the dissociation of the reactants, and an analysis has demonstrated that this is the case.

Thus of the different forms of glucose-1-phosphate, namely, undissociated glucose-1-phosphoric acid, and the mono- and di-valent ions $(C_6H_{11}O_5.O.PO_3H)^{-}$ and $(C_6H_{11}O_5.O.PO_3)^{=}$, on the one hand, and the different forms of orthophosphate, namely H_3PO_4, $(H_2PO_4)^{-}$, $(HPO_4)^{=}$, $(PO_4)^{\equiv}$, on the other hand, there is no doubt that the equilibrium is determined by the concentrations of the di-valent ions in each case. This became clear when the proportions of these different forms of the two substances existing at different concentrations of hydrogen ion were calculated, making use of the dissociation constants determined by Cori *et al.* (1937*a*); (for glucose-1-phosphoric acid $k_1 = 0{\cdot}78 \times 10^{-1}$; $k_2 = 0{\cdot}74 \times 10^{-6}$, and for orthophosphoric acid $k_1 = 1{\cdot}07 \times 10^{-2}$; $k_2 = 1{\cdot}51 \times 10^{-7}$, and (from Britton 1929) $k_3 = 2{\cdot}7 \times 10^{-12}$). Only on the assumption that the concentrations of the di-valent ions determine the equilibrium was a constant obtained.

This will be clear from the data given in table 7. By means of the calculated ratios: $(HPO_4)^{=}$/total orthophosphate, and $(C_6H_{11}O_5.O.PO_3)^{=}$/total glucose-1-phosphate for the different pH values, which are set out in the table, the value of $(HPO_4)^{=}/(C_6H_{11}O_5.O.PO_4)^{=}$ corresponding to the observed ratio *free-P/ester-P* at each pH value was derived. It will be seen that these values, $(HPO_4)^{=}/(C_6H_{11}O_5.O.PO_3)^{=}$, were constant within the limits of the experimental error.

It is clear that in the equilibrium state established at any pH value within the range examined the concentration of $(HPO_4)^=$ ion was 2·2 times that of $(C_6H_{11}O_5.O.PO_3)^=$ ion.

EFFECT OF VARYING CONCENTRATIONS OF GLUCOSE-1-PHOSPHATE AND ADDITIONS OF INORGANIC PHOSPHATE

In table 8 are given the results of an experiment in which the initial concentration of glucose-1-phosphate was varied from 25 to 122 mg. per 10 ml.; to some digests of the series inorganic phosphate was also added initially and, to accelerate the reaction, a small amount of starch.

TABLE 8. THE EFFECT OF VARYING CONCENTRATIONS OF GLUCOSE-1-PHOSPHATE AND INORGANIC PHOSPHATE

Digests 44–49. 10 ml. containing 2·5 ml. purified potato phosphorylase and additions of glucose-1-phosphate and in some digests phosphate buffer giving the initial amounts of *ester-P* and *free-P* stated; N/24 maleate buffer; final pH 6·05–6·20. Digest 50, 10 ml. containing 1·4 ml. purified phosphorylase (a different preparation) and no maleate; final pH 6·1.

	Initial amounts mg./10 ml.			Time (min.)						Final conversion
Digest	*ester-P*	*free-P*	Starch	0	5	12	25	50	90	%
44	2·08	0·01	—	**0·00**	—	**0·105**	**0·28**	**0·75**	**0·865**	86·5
45	2·08	0·01	12·5	**0·00**	**0·46**	**0·775**	**0·815**	**0·855**	—	85·5
46	4·16	0·01	2·5	**0·00**	**0·205**	**0·41**	**0·685**	**0·81**	**0·87**	87
47	2·08	3·72	2·5	**0·64**	**0·71**	**0·765**	**0·80**	**0·865**	**0·875**	65
48	2·08	6·96	2·5	**0·77**	**0·79**	**0·83**	**0·84**	**0·86**	**0·85**	37
49	3·12	14·7	2·5	**0·825**	—	**0·83**	**0·85**	**0·875**	**0·86**	19
50	1·04	10·5	—	**0·91**	—	—	**0·905**	—	**0·90**	—
				Time (min.)						
				0	17	74	186	419	1080	
51	11·1	0·03	—	**0·00**	**0·02**	**0·15**	**0·44**	**0·80**	**0·86**	86

(The figures in heavy type are values of the ratio: *free-P*/*free-* + *ester-P*.)

In digests 44, 45, 46 and 51 which contained no *free-P* initially the reaction proceeded in each case until the ratio *free-P*/*free-* + *ester-P* attained a value of about 0·86; the final equilibrium values were thus not significantly affected by the approximately fivefold variation in concentration of glucose-1-phosphate.

Again in digests 47, 48 and 49, to which different amounts of *free-P* were added initially with glucose-1-phosphate, approximately the same final values of the ratio were attained. In these cases the initial values of the ratio had been already increased by the additions of *free-P*, the initial value in one case approaching the equilibrium value (digest 49); under these conditions the equilibrium state was attained when a considerably smaller proportion of glucose-1-phosphate had been converted into starch and *free-P*. Values for the final percentage conversion of the added glucose-1-phosphate in each digest are given in the final column (table 8); these represent the observed increases in *free-P* expressed as a percentage of the initial amounts of *ester-P*.

It may be pointed out that no reaction occurs where inorganic phosphate and glucose-1-phosphate are added initially in such proportions that the ratio *free-P*/*free-* + *ester-P* is equal to or greater than the value characteristic of the equilibrium state at the prevailing concentration of hydrogen ions. (This is true only in the absence of starch; when starch is present, the ratio falls to the equilibrium value due with formation of more glucose-1-phosphate.) Digest 50 provides an example of the complete inhibition of the reaction when the initial ratio had been adjusted to 0·91.

It deserves mention that the final equilibrium state is not detectably affected by considerable variations in the concentration (or activity) of the added phosphorylase. Special experiments have been carried out to test this point but it seems unnecessary to describe these in detail since the experiments already given in tables 1–5 and 8 provide examples of digests of which the final pH values were approximately 6·1 and in which the volumes (and activities) of enzyme varied considerably; the equilibrium value of the ratio *free-P*/*free-* + *ester-P* in all these cases was close to 0·86.

RELATION BETWEEN AMOUNTS OF STARCH FORMED AND INORGANIC PHOSPHATE LIBERATED

In the reaction: glucose-1-phosphate → starch + *free-P*, the liberation of 1 mg. *free-P* would be accompanied theoretically by the formation of 5·24 mg. starch. In several large-scale experiments the yields of synthetic polysaccharide isolated at advanced stages of the reaction showed close agreement with this value. In the ensuing experiment the relationship was followed throughout the reaction.

Digest 52 consisted of 400 mg. glucose-1-phosphate, N/24 maleate buffer, and 5 ml. of phosphorylase solution (containing 27 mg. protein) in a total volume of 60 ml. The pH value was 6·0 initially and 6·2 finally.

In addition to the normal determinations of *free-P* and *free-* + *ester-P*, the formation of starch was followed by three methods. (*a*) Digest samples (5 or 3 ml.) were added to 5 ml. 0·5 % iodine solution; the precipitate was separated on the centrifuge in graduated tubes and its volume noted (in order to apply a correction for the small amount of glucose-1-phosphate included). The precipitate was suspended in 5 ml. water; the iodine was then removed by adding a layer of benzene and warming with gentle shaking. Hydrochloric acid to give a concentration of N/1 was added and the solution was held at 100° for 2·5 hr. The hydrolysate was then neutralized and diluted to 25 ml. and the reducing power was determined by a reagent of the Somogyi (1937) type. Under these conditions native starch and purified specimens of the synthetic starch yielded 104–106 % of their weight of glucose. (*b*) Samples of 1 ml. of digest for measurements of the iodine coloration were delivered into 1 ml. N soda. Later these were diluted, neutralized, and 5 ml. 0·1 % iodine (in 0·15 % potassium iodide solution) followed by water to 50 ml., were added. The iodine coloration was then measured by the approximate spectro-photometric method previously described (Hanes and Cattle 1938). The first sample (0·7 min.) gave extinction coefficients identical with the values observed on a blank solution containing the iodine only. From the observations on later samples were plotted a series of extinction/λ curves showing the increase in the extinction coefficient for each wave-length as compared with the corresponding value for the iodine blank. These curves were closely similar in form throughout the reaction; the maximum extinction was in the orange (mean filter transmission 610 mμ). For the present purpose these maximum values (E_{61}) alone will be given. (*c*) Finally, the proportion of the glucose engaged in ester and maltosidic linkages, respectively, was determined on 2 ml. samples which were delivered into 3·5 ml. hydrochloric acid (final concentration N/1). One set of samples was held at 60° for 30 min., while a parallel set was heated 2·5 hr. at 100°; these were neutralized and brought to 25 ml., and determinations of reducing power were made on suitable aliquots. By the former treatment glucose-1-phosphate is completely hydrolysed but only 2–3 % of the maltosidic linkages of starch were cleaved, whereas the latter treatment results in the hydrolysis of 95–97 % of the linkages of starch. From these data the extents of conversion of "ester linkages" into "maltosidic linkages" were calculated.

The results, which are presented in table 9, show clearly the parallelism between the formation of starch and the liberation of *free-P* throughout the reaction. The direct determinations of starch (by precipitation and subsequent hydrolysis) agree within the experimental error with the values calculated from the *free-P* values. Similarly the iodine coloration bears a constant relationship throughout the reaction to the liberation of *free-P*, and the ratio E_{61}/mg. *free-P* is close to that expected from observations on the iodine coloration of purified specimens of the synthetic polysaccharide (cf. p. 202). Finally the generation of resistant glucosidic linkages runs parallel with the liberation of *free-P*.

TABLE 9. RELATION BETWEEN STARCH FORMED AND FREE-P LIBERATED IN DIGEST 52

Method *a*

	Time (min.)						Calculated value
	0	20·5	50	114	172	265	
Increase in *free-P* mg./10 ml.	—	0·32	1·20	3·88	4·70	4·86	
Starch mg./10 ml.	0	1·61	6·80	19·8	23·1	24·6	
Ratio starch/*free-P*	—	**5·05**	**5·65**	**5·10**	**4·95**	**5·05**	5·24

Method *b*

	Time (min.)						Calculated value
	0	22·5	51·5	113	171	263	
Increase in *free-P* mg./10 ml.	—	0·38	1·26	3·87	4·70	4·86	
Iodine colour index E_{61}	0	0·12	0·47	1·37	1·62	1·65	
Ratio E_{61}/*free-P*	—	**3·25**	**3·73**	**3·54**	**3·44**	**3·40**	3·30*

Method *c*

	Time (min.)					
	0	22·5	51·5	115	175	267
Conversion *ester-P* to *free-P* %	0	6·5	22	68	85	86
Conversion ester linkages to maltosidic linkages %	0	5·8	22	66	82	84

* The E_{61} value observed with purified synthetic starch was 0·315 per mg. starch in 100 ml. (cf. p. 202). Hence calculated value of the ratio is $(2 \times 0{\cdot}315 \times 5{\cdot}24) = 3{\cdot}30$.

PREPARATION OF GLUCOSE-1-PHOSPHATE BY THE USE OF POTATO PHOSPHORYLASE

Specimens of glucose-1-phosphate have been prepared from starch and inorganic phosphate by the action of both crude and purified preparations of potato phosphorylase. For the large-scale preparation of the ester crude juice pressed from potatoes has been used successfully, the yields of 7–10 g. per litre of digest liquid being higher than those obtained in preparations using the pea enzyme described earlier (Hanes 1940*a*). Since the method of isolating the ester has been modified, an outline of the procedure will be given.

Digest 53 of which the total volume was 8·5 l. was made up as follows: enzyme, 850 ml. crude potato juice (freshly pressed); starch, 76 g. (dry basis) potato starch in the form of starch paste (prepared by washing the raw starch in 4 l. boiling water and holding at 100° for 20 min. with stirring); phosphate, 2·125 l. M phosphate buffer at pH 6·8. The initial concentration of added phosphate was thus 0·25 M. The progress of the reaction was followed by determinations of *free-P* and *free-+ester-P* on suitable samples; after 24 hr. 8·35 g. *free-P* had been converted into *ester-P*, and after 48 hr. 8·65 g., corresponding to the formation of 104 g. of the crystalline potassium salt of glucose-1-phosphate.

At this time the digest was killed by heating in 2 l. portions to 100°. The bulk of the inorganic phosphate was then precipitated by the addition of the calculated quantities of magnesium acetate and ammonia. The filtrate was reduced to 4 l. by the method of freezing previously described (reduction by vacuum distillation can be used). The remaining inorganic phosphate was then precipitated by adding a slight excess of ammonia (to pH 8·3) and removed by filtration.

The barium salt of the ester was now precipitated from the filtrate by adding 85 g. barium acetate (cryst.) followed by 7 l. methylated spirits, and after 15 hr. was removed on the centrifuge. The crude barium salt was suspended in 1 l. water and decomposed by adjusting the pH value to 1·8 with sulphuric acid; the precipitated barium sulphate was removed on the centrifuge and extracted three times by grinding with 150 ml. portions of water, followed by centrifuging. The combined supernatant solutions were brought to pH 8·3 with potassium hydroxide and 1·2 vol. methylated spirits were added.

After 24 hr. the crude potassium salt of the ester, still contaminated with dextrins and protein, was removed on the centrifuge, suspended in 1 l. water and brought to pH 5·5 by the addition of acetic acid. α-Malt-amylase (200 mg. of a purified preparation*) was now added in a little water; after 2 hr. the red-violet staining dextrins had been degraded to achroic, alcohol-soluble fragments. Trichloracetic acid in 50 % solution was now added to give a concentration of 5 % and then 1·4 vol. 95 % ethanol. A small precipitate consisting of protein and crystalline potassium acid sulphate was removed and discarded. The filtrate was brought to pH 8·3 with potassium hydroxide; crystallization of the potassium salt of the ester, which began immediately, was allowed to proceed 24 hr. at 0° when the product was filtered off and dried *in vacuo* over calcium chloride.

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* Flour from malt, which had been dried at 35° C, was suspended in four times its weight of water. The mixture was heated quickly to 70° C and, with efficient stirring, held at this temperature for 15 min. After rapid cooling, solids were removed by gravity filtration. A portion of the filtrate could be used for the purpose in question, but a dry preparation of α-malt-amylase (obtained from such a filtrate by precipitating the enzyme with 60 % alcohol, redissolving it in water and reprecipitating it with 80 % alcohol) was used in this case.

The crystalline product (88 g.), contained no *free-P* and 7·15 g. *ester-P* (i.e. 82·5 % of the *ester-P* present in the original digest); the content of *ester-P* was thus 8·12 % (as compared with 8·33 % theoretical). The product was further purified by dissolving it in 1 l. water and treating the solution with 3 g. charcoal at 50° C which removed the small amount of coloured material present; after filtering off the charcoal, 1 l. hot acetone was added, and crystallization was allowed to proceed for 24 hr. at 20° and then for several days at 0° C, a further 500 ml. acetone being added after 2 days.

After drying *in vacuo* over calcium chloride, the recrystallized product (79 g.) gave the following analytical values: rotation $[\alpha]_D^{21} + 78{\cdot}5°$ ($c = 1{\cdot}24\ \%$, $l = 4$ dm.); moisture loss (at 100° *in vacuo*, 1 mm., over P_2O_5) 9·70 %; apparent *free-P* (9 min. contact with acid reagent) 0·063 %; *ester-P* 8·35 %; *total-P* (after combustion) 8·35 %; C 19·5 %, H 4·10 % (according to Pregl by Dr G. Weiler); residue (after 2 hr. at 1000° C) 44·0 %. ($C_6H_{11}O_5.O.PO_3K_2.2H_2O$ requires 9·67 % moisture, 8·33 % P, 19·35 % C, 4·06 % H and 44·3 % residue as $K_4P_2O_7$.)

This product was indistinguishable from purified specimens of the ester formed by the action of pea phosphorylase. (It should be mentioned that redeterminations of the rotation of these have given slightly higher values than that reported earlier, $[\alpha]_D^{21} + 78$–$79°$ instead of $+76{\cdot}5°$.)

ISOLATION AND PROPERTIES OF THE SYNTHETIC POLYSACCHARIDE

Several specimens of the enzymically synthesized starch, 22 g. in all, have been isolated and purified by different methods.* An outline of the procedure adopted in one of these experiments will suffice for the present purposes.

Starch specimen A from a large digest (54) which consisted of 150 ml. purified phosphorylase solution (containing 1·1 g. protein), 25 g. glucose-1-phosphate (dissolved in water and adjusted to pH 6·0 with acetic acid) and water to make 2 l. At intervals during the reaction the pH was re-adjusted to 6·0 by dropwise additions of N acetic acid, 26 ml. in all being required.

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* Since this was written a large specimen of the starch, amounting to 55 g., has been prepared for constitutional study.

After 5·5 hr. the increase in *free-P* was 1·745 g., indicating the conversion of 83 % of the glucose-1-phosphate and the formation theoretically of 8·98 g. starch. The reaction was now arrested by adding 200 ml. 1 % iodine solution (in 1·5 % potassium iodide). After 15 hr. the resulting blue-black precipitate was removed on the centrifuge; the supernatant solution which contained only a trace of iodine-staining material was discarded. The iodine was removed from the precipitate by hot treatments with ethanol, acetone and finally benzene.

The crude product (9·4 g. dry basis) contained 8·2 g. starch (determined by the hydrolysis of a small sample—2·5 hr., 100°, N/1 hydrochloric acid) or 91 % of the theoretical amount. It contained 0·18 g. total nitrogen indicating that the contaminant (1·2 g.) was mainly protein. Considerable purification was now effected by dissolving the starch in hot neutral calcium chloride solution (sp.gr. 1·32), an insoluble fraction remaining after five extractions being discarded. The starch was precipitated from the combined extracts with 70 % ethanol; it was suspended in warm water and reprecipitated. After washing with ethanol (70, 95 %, and absolute) the product was dried—yield 8·85 g. (dry basis) containing 0·4 % N and about 5 % ash.

A portion of this material (6·1 g. dry basis) was suspended in 100 ml. cold water; 10 N sodium hydroxide was added slowly with stirring. When 6–7 ml. had been added the suspension became clear; with the addition of 40 ml. a small precipitate formed which did not increase on further additions. This was removed on the centrifuge and the clear supernatant solution was neutralized to pH 7 with glacial acetic acid (added dropwise with cooling) and a further small precipitate which formed was removed. The product was precipitated from the supernatant solution with 70 % ethanol and after two reprecipitations it was washed in absolute ethanol and acetone and dried *in vacuo*. The yield of the purified material (5·2 g. dry basis) after correcting for an ash content of 2·9 % was equivalent to a 75 % recovery of the starch synthesized in the original digest.

The following analytical values, expressed on the basis of moisture- and ash-free material, were observed with this specimen: N 0·026 %; total P 0·028 %; *reducing power* (by copper method) 1·0 (glucose = 100); *reducing power after hydrolysis* (2·5 hr., 100°, N hydrochloric acid) 104 (as compared with 105–106 found for native potato starch under the same conditions); *rotation* $[\alpha]^{21}_{D}$ + 202° in water ($c = 0·2$ %), + 158° in 5 % soda ($c = 0·3$ %); C 45·4 %, H 6·45 % (according to Pregl by Dr G. Weiler). $C_6H_{10}O_5$ requires 44·5 % C, 6·23 % H.

In the above characteristics the product was indistinguishable from natural starch. Certain of these observations have been made on several other specimens of the synthetic material; these have shown some slight variations in reducing power, the values ranging from 0·5 to 1·2 (glucose = 100) and in rotation, the $[\alpha]_D^{20}$ values ranging from 197 to 212°. These differences may have reflected differences in the methods and extent of purification.

In certain respects all specimens of the enzymically synthesized polysaccharide so far prepared have differed significantly from natural potato starch.

In the first place must be emphasized the sparing solubility of the synthetic product in water and the rapidity with which it retrogrades from solution. If the dry material is rubbed in a mortar in the form of a thick paste and is then washed into boiling water, a clear solution, stable on subsequent cooling, is obtained only when the final concentration is less than about 0·1–0·2 %; with higher concentrations an amorphous deposit of retrograded starch is formed in a short time. The product is readily soluble in strong alkalis, a 3 % solution in N sodium hydroxide, for example, being clear and stable. On neutralization a considerable portion of the synthetic starch is deposited from solution after periods varying from a few minutes to several hours, depending on the concentrations of starch and salt (formed on neutralization). A clear solution may be obtained by autoclaving (e.g. 30 min. at 120° C) but again the material retrogrades except in dilute solutions.

TABLE 10. QUANTITATIVE OBSERVATIONS ON IODINE COLORATION

	Values of $E - E_{\text{iodine}}$/mg. polysaccharide per 100 ml.								
	Mean filter transmission (A units × 10^2)								
	43	47	50	53	57	61	66	72	75
Synthetic starch	0·08	0·115	0·11	0·19	0·29	**0·315**	0·27	0·16	0·13
Natural starch*	0·05	0·08	0·10	0·12	**0·15**	0·145	0·12	0·08	0·06
Amylo-amylose†	0·08	0·105	0·12	0·16	0·23	**0·26**	0·22	0·15	0·12

* Values for potato starch from Hanes and Cattle (1938).

† Prepared in 1936 by the method of Samec and Mayer (1921); stored as dry powder.

A second point of difference lies in the colour developed on the addition of iodine to solutions of the synthetic product; as judged visually, thus this is more brilliant blue than the colour given by natural starch. Quantitative measurements have not only confirmed this difference in the hue but have shown also that the intensity of the colour developed is much greater than that given by natural starch. In table 10 are given the values for the increase in extinction as compared with the iodine solution alone (10 mg. iodine + 15 mg. potassium iodine per 100 ml.) resulting from the presence of 1 mg. starch per 100 ml. These values $E - E_{\text{iodine}}$/mg. for all wave-lengths, were unaffected by variations in the concentration of starch between 1 and 10 mg. per 100 ml. For comparison, the values observed with soluble potato starch under the same conditions (from Hanes and Cattle 1938) are given.

It will be seen that the position of maximum extinction exhibited by the iodine complex of the synthetic material is slightly nearer the red end of the spectrum, and that the extinction values are considerably higher, particularly in the longer wave-lengths, than those observed with natural starch. The latter values, it should be stated, represent the highest so far observed amongst a number of starch specimens which have shown a variability of about 15 %.

The third peculiarity of the synthetic polysaccharide became evident during a study of its degradation under the action of different amylases. With dextrinogenic amylases the behaviour was similar to that observed for natural starch; under the action of α-malt-amylase, for example, the iodine colour was destroyed when approximately one-sixth of the glucosidic linkages had been converted into reducing groups.

The abnormality of the synthetic polysaccharide became evident only from its behaviour under the action of purified β-amylase (from ungerminated barley). In the case of natural starches of several varieties, it is recognized that when approximately 60 % of the starch substance has been converted into maltose by this enzyme the reaction ceases and there remains a resistant fragment (α-amylodextrin) which exhibits a blue-violet coloration with iodine. The mechanism of this process has been discussed elsewhere (Hanes 1937). It has been found that the enzymically-synthesized starch exhibits no such obstruction to the action of β-amylase, the reaction proceeding smoothly until 95–100 % of the polysaccharide has been converted into maltose; the iodine coloration remains blue (in diminishing intensity) up to the stage of 80–85 % conversion when it turns to pale rose which persists until 90–95 % conversion.

While the full discussion of this peculiarity of the synthetic starch will be undertaken elsewhere attention is drawn to the fact that the amylo-amylose fraction of natural starch (isolated by the method of Samec and Mayer 1921) exhibits identical behaviour under the action of β-amylase (cf. Samec 1936; Freeman and Hopkins 1936; Hanes 1937). Furthermore, amylo-amylose closely resembles this product not only in its low solubility and tendency to retrograde from solution, but also in the colour it exhibits with iodine (cf. table 10). There is little doubt, in fact, that the enzymically synthesized material is extremely closely related to this particular component of natural starch. The considerable interest which attaches to this conclusion lies in the possibility now opened of elucidating the still unexplained differences in the behaviour of physically separable components of native starch.

THE BEARING ON PROBLEMS OF PLANT METABOLISM

A brief comment may be made on the implications of the present results on questions of the metabolism of carbohydrates in the plant.

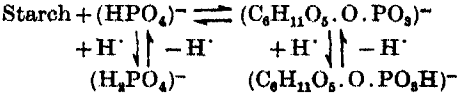
The biochemical setting of the reversible transformation here investigated is indicated in the schema on p. 175 in which are represented the related reactions which were shown to occur in extracts of peas. Adopting this picture which is known to be incomplete, and anticipating the rigid proof that starch formation in the plastid of the plant cell is due to the phosphorylase mechanism, it is evident that glucose-1-phosphate occupies a position of central metabolic importance. Not only is this ester the direct precursor of starch, but it forms the initial reactant in the sequence of interconversions leading to the formation of fructose-diphosphate. According to a view for which evidence is accumulating, these latter reactions represent the preliminary steps in the catabolism of hexose in the plant cell. In this case the reversible formation of starch from glucose-1-phosphate may be regarded as a side reaction, incidental to the essential processes of carbohydrate breakdown associated with respiration.

It must be assumed that there exists in the cell a mechanism by which the primary synthesis of glucose-1-phosphate is effected from hexose (or some simpler compound) together with inorganic phosphate. In this sense the generation of glucose-1-phosphate by the "phosphorolysis" of starch under the action of phosphorylase is to be regarded as a secondary mechanism by which the ester can be reformed readily. Starch might be said to constitute an accessible supply of hexose-phosphate, a conception which clarifies the traditional statement that starch forms a reserve of "readily available" carbohydrate for the plant.

CONCLUSIONS

The foregoing experiments establish that phosphorylase from potato tissue catalyses both the formation of glucose-1-phosphate from starch and inorganic phosphate and the reverse process. The reversibility of the transformation is shown by the fact that the reaction proceeds in either direction until the ratio *free-P/ester-P* attains a value which is not significantly altered by wide variations in the concentrations of the reactants or the enzyme.

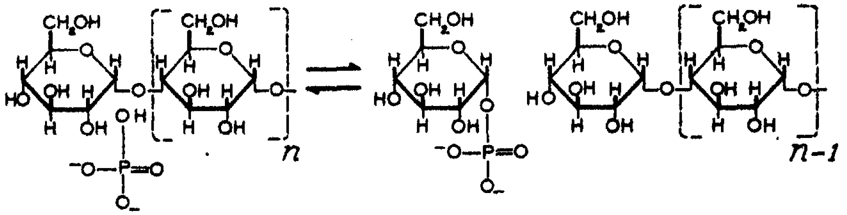
The equilibrium values of this ratio vary considerably, however, with the concentration of hydrogen ion. This has been shown to be due to the fact that the equilibrium is governed by the relative concentrations of the di-valent ions only of orthophosphoric acid and glucose-1-phosphoric acid and not by the total concentrations of inorganic phosphate and glucose-1-phosphate. Thus when the pH value was varied from 5 to 7 the observed values at equilibrium of the ratio *free-P/ester-P* (i.e. total inorganic phosphate/total glucose-1-phosphate) diminished progressively from 10·8/1 to 3·1/1 whereas the ratio $(HPO_4)^{=}/(C_6H_{11}O_5.O.PO_3)^{=}$ remained constant at 2·2/1 (± 5 %). The influence of variations in the hydrogen ion concentration upon the equilibrium is thus due to its effects upon the dissociation of inorganic phosphate and glucose-1-phosphate as indicated in the following schema (in which ionic forms present only in traces in the pH range 5–7 are not included):



$$\begin{array}{ccc} \text{Starch} + (HPO_4)^{=} & \rightleftharpoons & (C_6H_{11}O_5.O.PO_3)^{=} \\ +H^{\cdot}\ \downarrow\uparrow\ -H^{\cdot} & & +H^{\cdot}\ \downarrow\uparrow\ -H^{\cdot} \\ (H_2PO_4)^{-} & & (C_6H_{11}O_5.O.PO_3H)^{-} \end{array}$$

A point of interest is that the equilibrium is not perceptibly affected by alterations in the concentration of starch. This would seem to indicate that the *effective* concentration of starch is independent of the total concentration. A possible explanation may be that the phase of the colloidal system in which the reaction occurs is maintained in a state of saturation in respect to a form of starch in true solution or in a highly disaggregated condition. This hypothesis might be tested by determining the effect of additions of low-molecular disaggregated dextrins which, as was shown earlier, form suitable substrates for the phosphorylase of peas.

With regard to the mechanism underlying the interconversion, all the known facts are accommodated most readily by the hypothesis that the formation of glucose-1-phosphate from starch and inorganic phosphate is the result of the direct "phosphorolytic" cleavage of successive terminal glucose units from the chain-molecules of starch and, conversely, that the formation of polysaccharide from the ester is the result of a succession of "de-phosphorolytic" condensations. This conception which was advanced by Parnas (1937) and Cori, Colowick and Cori (1937*b*, 1938) to explain the formation of glucose-1-phosphate from glycogen under the action of muscle phosphorylase may be represented schematically as follows:



While the separate stages of the reaction in either direction find a plausible explanation in this conception it must be emphasized that important aspects of the interconversion require further experiments for their elucidation. A hint of the possible complexity of the process which leads to the elaboration of the complex molecule of starch from glucose-1-phosphate is afforded by the phenomenon of the initial lag in this reaction, a feature which suggests that the initial stage in the condensation process may be different from the subsequent stages. The fact that additions of starch or maltose accelerate the reaction, and shorten or abolish the induction phase, might be considered to support this view; it is possible that the presence of compounds containing the maltose grouping may be essential for the initiation of the reaction, and the activating effect of these substances may be due to their direct participation in the early stages of the condensation process. This and alternative possibilities will be considered in a later communication.

An investigation of the molecular constitution of the enzymically synthesized starch, which is being undertaken, may be expected to establish the uniformity or otherwise of the product, and whether it is made up of the recurrent chemical units, comprising chains of 24–30 glucose units, which have been found by Haworth, Hirst, and various collaborators (cf. review, Hanes 1937) to characterize the range of varieties and fractions of natural starch hitherto investigated. Any discussion of the questions whether the condensation process occurring in this enzymic system leads to the formation of chains of uniform length and, if so, whether this occurs in one or several stages must be deferred until the results of this study are available.

In the meantime, as has been mentioned earlier, the physical properties of the synthetic polysaccharide, the intensity of the blue colour it gives with iodine, and the fact that it is hydrolysed almost to completion by β-malt-amylase suggest that it is related more closely to the amylo-

amylose fraction of starch than to normal whole starch. The differences in properties exhibited by this physically separable fraction of the starch substance have been emphasized earlier (Hanes 1937) and it becomes increasingly desirable that it be examined by the modern constitutional methods.

The formation of well formed grains of the synthetic starch has been mentioned (p. 183) as a feature which invites detailed investigation.

An important question which emerges is the relationship between the phosphorylase from higher plants and the corresponding enzymes from animal tissues and yeast. Preparations of the latter, in varying degrees of purity, have been reported to catalyse the formation of glycogen from glucose-1-phosphate. It appears that the muscle phosphorylase described by Cori, Schmidt and Cori (1939) and Cori and Cori (1939) was practically free from both amylase and the phosphoglucose-conversion enzymes. It is noteworthy that the polysaccharide formed by the action of this preparation exhibited a blue colour with iodine. Thus, although this product was referred to as glycogen by Cori *et al.*, there is a suggestion that it may be more closely related to starch than to glycogen; on the basis of the observations so far available it could not be distinguished from the polysaccharide formed by the action of potato phosphorylase. It seems likely that further study of these products may clarify the underlying relationship between starch and glycogen.

It is appropriate to mention in conclusion the prospect that future investigations in this general field will elucidate the range of processes, chemical and physical, which are involved in the building up from simple substances of the complex organic matter which forms biological structure.

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The structure of 'ineffective' nodules and its influence on nitrogen fixation

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[Plates 13, 14]

1. The anatomy and cytology of nodules produced on clover, peas and soy beans by 'effective' and 'ineffective' strains of *Rhizobia* were investigated, with especial reference to the changes in volume of the active infected tissue during the life of the nodule.

2. In clover the mean volume of this active bacterial tissue is about three times as great in 'effective' as in 'ineffective' nodules. This is due to an early arrest of growth in nodules produced by ineffective strains.

3. In all nodules the active bacterial tissue eventually disintegrates, but in effective clover nodules it remains without disintegration for about six times as long as in ineffective nodules.

4. In an experiment to test the nitrogen fixation by clover inoculated with an effective and an ineffective strain, the difference between the strains in the amounts of nitrogen fixed could be accounted for by the differences in volume and in duration of the active bacterial tissue.

5. In peas, nodules produced by an effective strain were nearly twice the

length of those produced by an ineffective strain, and their bacterial tissue remained without disintegration for about twice as long.

6. In soy beans the mean volume of bacterial tissue was 4·75 times as great in effective as in ineffective nodules and the percentage of that volume composed of infected cells was twice as great.
7. In ineffective soy bean nodules disintegration of the bacterial tissue began when the plant was 4 weeks old and was practically complete by the twelfth week, at which time no disintegration could be found in effective nodules.
8. The difference in amount of nitrogen fixed by soy bean plants bearing each type of nodule could be accounted for wholly by the factors mentioned above.
9. Thus in both clover and soy bean nodules the volume and duration of the active infected tissue are the main, if not the only, factors determining differences in nitrogen fixation amongst the strains tested.

A. INTRODUCTION

The fact that strains of nodule bacteria differed in their ability to benefit the host legume was realized as early as the nineties of last century. It was, however, the careful work of Stevens (1925) working with lucerne, and of Wright (1925*a*, *b*) with soy beans, that definitely showed the wide differences in nitrogen fixed in the same species of host legume when infected with different strains of bacteria. Their work has been amply confirmed and has been extended to other host plants by numerous workers such as Helz, Baldwin and Fred (1927) working with peas, Eckhard, Baldwin and Fred (1931) with lupins, and Baldwin and Fred (1929) with clover.

In some cases, especially amongst clover nodule bacteria, strains have been found that fix very small amounts of nitrogen and confer scarcely any benefit on their host. Such strains are here referred to as 'ineffective' although this term is relative and it is doubtful whether strains exist that fix no nitrogen at all.

Various authors have attempted to find some correlation between ineffectiveness and such other characters as serological behaviour and cultural features shown *in vitro*. Stevens (1925) and Wright (1925*a*) had some success in relating ineffectiveness to agglutination reactions, while Baldwin and Fred (1927) found that strains of lucerne nodule bacteria showed differences in respect to mannitol fermentation that agreed with the determinations of effectiveness in nitrogen fixation found by Stevens (1925). But, on the whole, little correlation has been found between effectiveness in the plant and behaviour of the organisms in laboratory culture.*

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* Dr Hugh Nicol tested the strains of nodule bacteria that form the subject of this paper as regards their growth and the change of reaction produced in media containing a wide range of carbohydrates and higher alcohols. No differences were found that could be correlated with effectiveness towards the host plant.

It has, nevertheless, been natural to suppose that bacteria of ineffective strains fix less nitrogen in the host plant owing to some defect in the mechanism of nitrogen fixation possessed by them. This is not self-evident, however, since crude nitrogen determinations of host plants inoculated with different strains do not provide a measure of the nitrogen-fixing efficiency of the bacteria unless an estimate can be made of the quantities fixed by unit masses of bacteria in unit time. To obtain this estimate, corrections must be made for such factors as the number of nodules, the volume of active tissue containing bacteria within these nodules, and duration of its activity. Adequate data for making even approximate corrections of the last two factors have not hitherto been available. The need for such data has, however, long been apparent from the frequently recorded fact that both the numbers and mean size of nodules show large differences as between strains, those that are ineffective tending to produce more numerous but much smaller nodules.

Surprisingly few observations have been recorded in which the anatomy and cytology of nodules produced by effective and ineffective strains have been compared. Elizabeth McCoy (1929) studied the anatomy of nodules on *Phaseolus*. Earlier workers such as Frank (1890) and Schneider (1892) considered that nodules on *Phaseolus* as a class were not beneficial to the host plant. Benefits from inoculation have been recorded with *Phaseolus* by Wilson and Leland (1929) but it does seem that ineffective strains are particularly prevalent with this host plant. In any case, the nodules studied by McCoy were apparently on an ineffective type. She found that the formation of the nodule was due rather to the multiplication of infected cells than to that of uninfected cells which were later invaded by the bacteria, as is more usual in legume nodules. The former process, however, has been found by Milovidov (1926, 1928) to be typical of lupin nodules, an observation confirmed by us, and is hence more likely to be conditioned by the type of host plant than by the particular strain of the invading organism. McCoy also found her *Phaseolus* nodules to be unusual in the following characters. (1) There were relatively few infected cells in the central tissue. (2) The nodules contained an abundance of starch associated with a great development of mitochondria. (3) The bacteria were rod-shaped and did not change into swollen or branched 'bacteroids'.

Marie Löhnis (1930) studied the anatomy of pea and clover nodules, in each case produced by an effective and an ineffective strain. She found that the two types of pea nodules differed in the amount and distribution of starch, the ineffective nodules containing more starch, more widely distributed through the nodule. Her effective nodules also contained a peculiar type of bacterial cell not found in the ineffective nodules. In clover nodules, however, she records no differences in anatomy, in amount and distribution of starch or in the shape of the bacteria as between effective and ineffective nodules. This similarity suggested that the unusual characters found in ineffective nodules on *Pisum* and *Phaseolus* are not necessarily associated with inefficiency.

It therefore seemed desirable to study the anatomy and cytology of nodules produced on several host plants by effective and ineffective strains, first, in order to determine what characters were common to ineffective but not found in effective strains, and, secondly, to obtain quantitative data from which it might be possible to form some estimate of the relative amount of nitrogen fixed by a unit mass of bacteria in unit time in nodules of each class. For without such an estimate it was impossible to determine how much of the ineffectiveness of a given strain was due to relative inability of the individual bacteria to carry out the process of nitrogen fixation, and how much to poor growth of the bacteria within the host tissues.

B. Material and methods

The nodules on clover, peas and soy beans were chosen for this investigation, because these are widely different types of host plant from which strains of nodule bacteria have been isolated that differ greatly in effectiveness. The following bacterial strains were used:

Soy bean		Supplied by the Wisconsin Agricultural Experiment Station.*
Effective strain	501	
Ineffective strain	507	
Pea		
Effective strain	310	
Ineffective strain	B. 33	
Clover		
Effective strain	205	
Ineffective strain	202	

Effective strain A, supplied by the Agricultural Experiment Station, Stockholm.

Ineffective strain Coryn, obtained from hill pasture on Coryn Mountain, Aberystwyth.

* The authors are gratefully indebted to Professor I. R. Baldwin, Wisconsin Agricultural Experiment Station, and to Professor Christian Barthel, Landbrukskogkolan, Uppsala, for supplying the cultures referred to; also to the Staff of Aberystwyth University for the clover plants from which the Coryn strain was isolated.

Strains 205 and 202 are the same that formed the subject of Marie Löhnis' observations on clover nodules.

The original strains 205 and 202, obtained from Wisconsin, lost their power of producing nodules during the course of the work, although strain 205 has since recovered this power without loss of effectiveness. In the meantime, however, a second active culture of each strain was obtained from Wisconsin in 1937, and these are here distinguished by the numbers 2057 and 2027.

The anatomical descriptions given below are based on the study of nodules on clover grown in agar and on peas and soy beans grown in sand.

The clover plants used for this study were grown in agar media in test tubes 1¼ in. in diameter. Three methods were employed. The first was the usual one of growing the plants in agar blocks made by pouring about 30 ml. of the melted agar medium into each tube and allowing it to solidify with the tube kept upright. Seeds were sown near the glass so that a good proportion of the root system should remain visible. The second method was to pour 10 ml. of melted medium into each tube and to solidify it by rotating the tube under a stream of cold water, as is done in the 'roll-tube' method of plating bacteria. Seeds were sown at the upper edge of the film of medium. Such cultures have the advantage that the whole root system remains visible through the glass, so that the appearance and growth of the individual nodules can be followed. It was used in obtaining growth curves of nodules and to obtain nodules of known age for anatomical study. The method suffered from a tendency of the agar film to dry up. This drying could be delayed by adding a few ml. of sterile water to each tube, but it was later replaced by a third method in which 12 ml. of agar medium were added to each tube and allowed to solidify and form a slope, the seed being sown at the upper end.

For the first, or agar block method, the following medium was employed:

K_2HPO_4	0·5 g.	NaCl	0·1 g.	$FeCl_3$	0·01 g.
KH_2PO_4	0·5 g.	$Ca_3(PO_4)_2$	2·0 g.	Agar	10·0 g.
$MgSO_4 . 7H_2O$	0·2 g.	$FePO_4$	0·5 g.	Tap water	1 l.

For the roll and slope cultures, the medium was modified as follows in order to stiffen the agar gel and to reduce the opaque phosphate precipitate:

K_2HPO_4	1·0 g.	$FeCl_3$	0·01 g.
$MgSO_4 . 7H_2O$	0·2 g.	Agar	20·0 g.
$CaH_4(PO_4)_2 . 2H_2O$	0·5 g.	Tap water	1 l.
NaCl	0·1 g.		

The tubes of agar were autoclaved for 20 min. at 15 lb. pressure. For inoculation, the tubes were cooled to 42° C and a loopful of the appropriate culture was added to the melted agar and mixed by gentle shaking. This method gives quicker and heavier infection of the plant than that of adding the bacteria after the agar has solidified.

The seeds were externally sterilized by shaking for 3 min. in absolute alcohol, for another 3 min. in 0·2 % $HgCl_2$ and washing in four changes of sterile water. Two to four seeds were planted in each tube with a flamed platinum loop. During growth, the tubes were supported in blocks of wood drilled with suitable holes of such a depth as to keep the root system shaded.

For nitrogen determinations, clover was grown in quart milk bottles each containing 800 g. of sand and 100 ml. of the following food solutions:

K_2SO_4	0·9 g.	$MgSO_4.7H_2O$	0·5 g.	Boric acid	0·02 g.
K_2HPO_4	0·5 g.	NaCl	0·5 g.	$MnSO_4$	0·02 g.
$CaH_4(PO_4)_2.2H_2O$	0·5 g.	$FeCl_3$	0·02 g.	Tap water	1 l.

The bottles of sand were autoclaved for 2 hr. and the food solution separately autoclaved for 15 min. at 15 lb. pressure. The inoculum was suspended in the food solution and the suspension added to the sand with a sterile pipette. Seven to ten externally sterilized seeds were sown in each bottle.

Peas and soy beans were grown in glazed earthenware pots, the former in small pots containing 3 kg., and the latter in large pots holding 12 kg. of sand. The pots of sand used for peas were rendered free from a contamination of nodule bacteria by blowing steam upwards through the sand for 14 min. via the hole at the base of the pot. In the case of soy beans the pots and sand were not sterilized, as these were found to be free from soy bean nodule bacteria.

The same food solution was used for peas and soy beans as for the bottle cultures of clover. 200 ml. of this solution was added to each of the small pots and 1000 ml. to each of the large pots. More food solution was added as required during the course of the experiments. The solution used in growing the peas was sterilized and watering was done with boiled water. The inocula were added to the first dose of food solution before adding this to the pots. Seeds of both peas and soy beans were externally sterilized and sown at a uniform depth. In all experiments with clover, peas and soy beans, uninoculated controls were grown and these remained free from nodules.

For anatomical study, nodules were fixed at intervals during the growth of the host plants and in the case of clover, nodules of known age were taken. They were usually fixed in Allen's modification of Bouin's solution (Allen's P.F.A. 3) and occasionally in Flemming's medium solution (as given by McCoy 1929). They were brought through alcohols into chloroform, imbedded in paraffin wax and cut into sections 6–10μ thick. Most of the sections were stained with Heidenhain's iron haematoxylin with or without a counter-stain of erythrosine or orange G.

For studying the distribution of starch, slides were treated with a mordant of 2% aqueous solution of tannin for 12 hr., stained for 2 min. with 1% aqueous gentian violet and differentiated in 95% alcohol. Excellent results were obtained when this method was combined with the haematoxylin stain. The slides were then treated as above described after the haematoxylin had been differentiated with iron alum, and were finally counter-stained with orange G.

C. THE DEVELOPMENT AND STRUCTURE OF CLOVER NODULES PRODUCED BY DIFFERENT STRAINS OF BACTERIA

In agar cultures of clover, the nodules induced by the strains here studied, first appeared on plants about 8 days old, which had produced their first true leaves. Nodules visible to the naked eye appeared on the same day in which infected root hairs were first observed, indicating that the bacteria pass down the root hair and induce proliferation of the root cells within 24 hr. The early development of the nodule follows the course described by Thornton (1930*a*) for lucerne nodules. The first stage consists of a mass of proliferating cells (figure 1, plate 13) mostly in the cortex but penetrating into the pericycle. The central cells soon swell and most of them become infected with bacteria brought into them by the infection threads (figure 2, plate 13). At this stage also, vascular strands begin to be differentiated along the sides of the nodule. By the time the nodule is a week old the cytoplasm of the infected cells in the central region becomes filled with bacteria. This central tissue is referred to below as the 'bacterial tissue' (figure 3, plate 13).

At the distal end of the nodule a cap of cells remains meristematic. In all clover nodules studied, the bacteria in recently infected cells close behind this meristem cap are rod shaped, but in older parts of the bacterial tissue they change into branched or swollen forms, often referred to as 'bacteroids'. The shape of these bacteroids differs considerably in different strains but these differences are not correlated with the effectiveness of the strain. The bacteroids in nodules produced by strains 2057, A and 2027 are usually pear-shaped or branched, an observation already made for the two

American strains by Marie Löhnis (1930). In the Coryn strain the bacteria undergo a type of change never observed by the authors in any other nodules (figure 4). Swellings appear either at the end or in the centre of the young rod-shaped cells and increase until they absorb the whole of the rod, which is thus converted into a sphere. At first the staining material is distributed throughout the spherical cell but later this became collected to form a deeply staining granule. Final disintegration releases these granules from the cells.

The amount and distribution of starch varies very greatly in individual clover nodules but seems to bear no relation to the effectiveness of the strain.

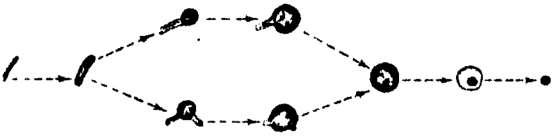

FIGURE 4. Formation of spherical forms by Coryn strain bacteria in the nodule.

The early course of nodule formation is common to all the strains studied, whether effective or ineffective. It results in the formation of an approximately spherical nodule about 0·5 mm. in diameter. From this stage, however, the course of events differs in effective and ineffective nodules.

In nodules produced by the effective strains 205, 2057 and A, the apical meristem continues its activity (figure 5, plate 13) and causes the nodules to grow in length so that, when 7 weeks old, they have a mean length of about 2 mm. (figure 6).* The mean length of such nodules of all ages on plants ten weeks old is 1·45 mm. In nodules produced by the ineffective strains Coryn and 2027, the meristem cap ceases to function after about 7 days so that the nodules remain small and round, having a mean diameter of 0·8 mm. In these nodules there is therefore a very small volume of bacterial tissue (figure 3, plate 13). The volume of this tissue in clover nodules bears a fairly constant relationship to their overall length, as was also found to be the case with lucerne (Thornton and Nicol 1936). This relationship, found for nodules of strain 2057 and Coryn, is shown graphically in figure 7, which is based on measurements of nodules from plants of varying age grown in agar and on the assumption that the bacterial tissue is cylindrical in shape. The mean length of strain 2057 nodules on plants 10 weeks old corresponds to a bacterial tissue volume of 0·22 cu. mm.,

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* The data plotted in figure 7 are derived from the agar cultures described below in § D (p. 218). The volume of organized bacterial tissue plotted in figure 10 were also obtained from these cultures.

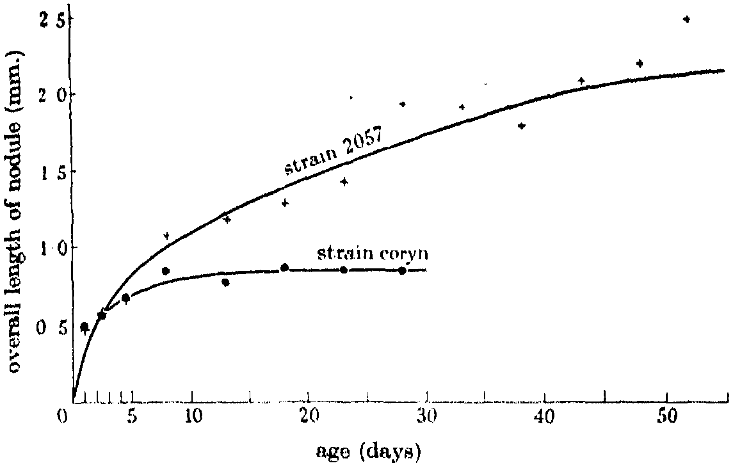


FIGURE 6. Growth in length of effective and ineffective clover nodules.

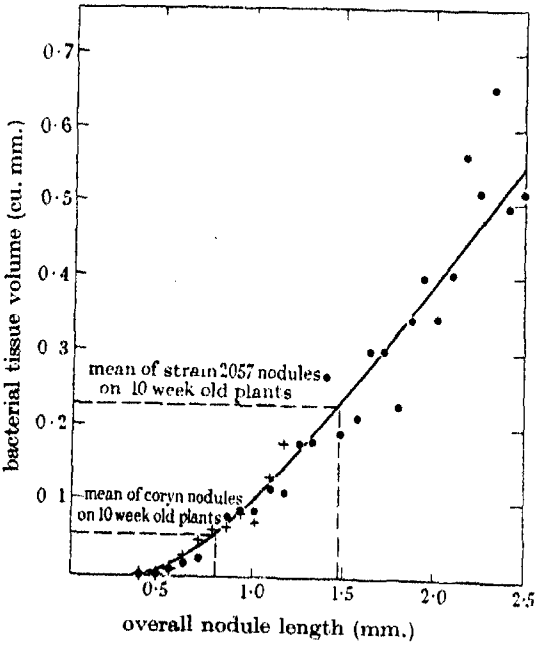
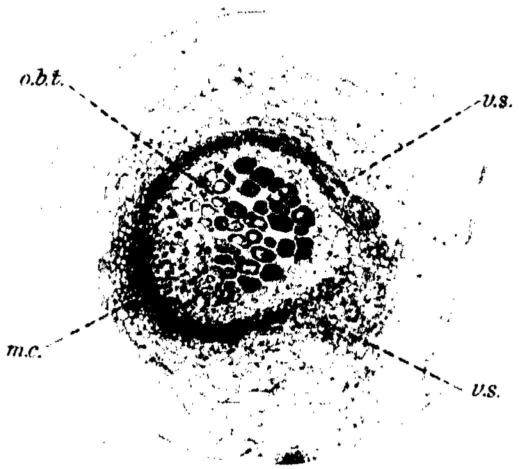


FIGURE 7. Relation of overall length to volume of bacterial tissue in clover nodules. Each point gives the mean volume of bacterial tissue in from 3 to 10 nodules of the same overall length. Dots refer to strain 2057, crosses to the Coryn strain nodules.



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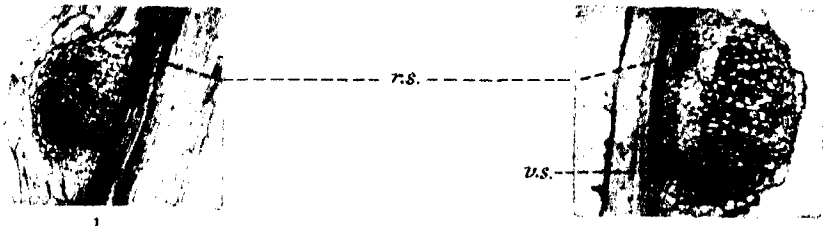

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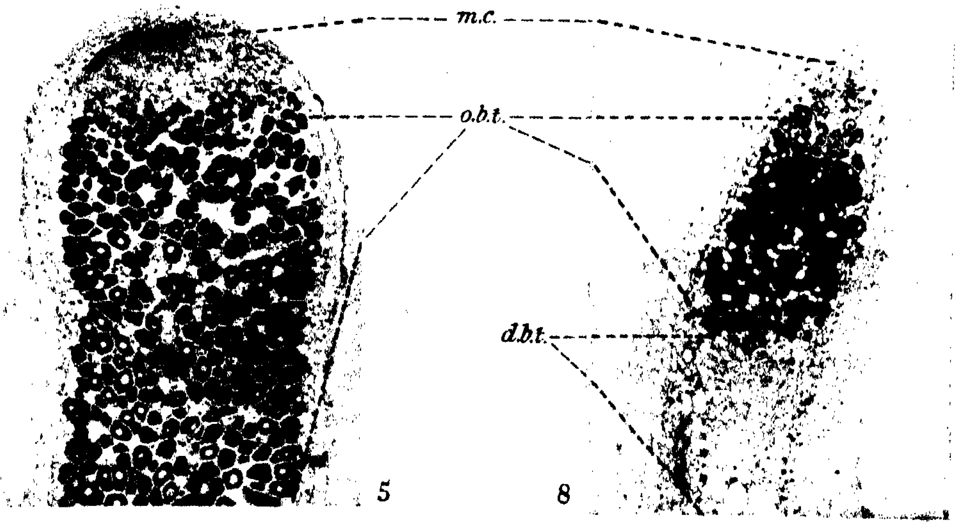

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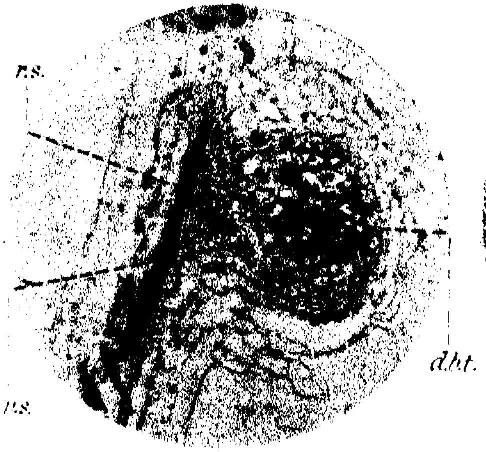

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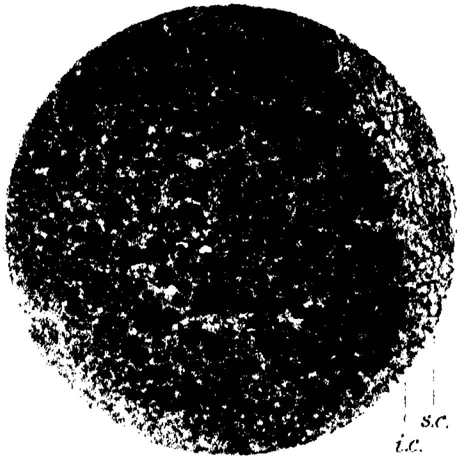

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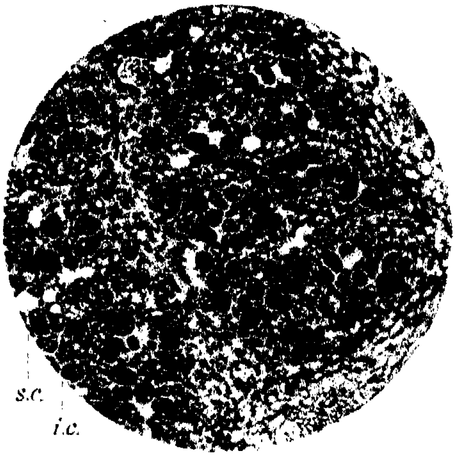

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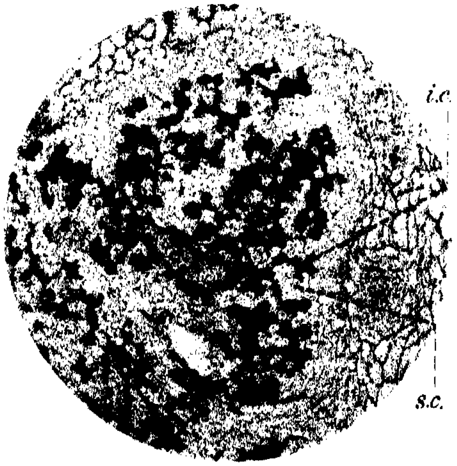

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whereas that of Coryn nodules corresponds to a bacterial tissue volume of only 0·05 cu. mm.

In clover nodules of all the strains studied, the bacterial tissue eventually disintegrates owing to parasitic attack on the tissues by the bacteria, as has been described in the nodules on clover and lucerne by Thornton (1930*b*). But one of the most striking differences between effective and ineffective nodules is the time at which this disintegration takes place. In nodules produced by the effective strains, the bacterial tissue begins to disintegrate at the base, when the nodule is about a month old (figure 8, plate 13). After about eight weeks the centre of the nodule usually shows complete disintegration and is filled with collapsed and necrotic cells. In ineffective nodules of strains 2027 and Coryn, disintegration begins when the nodule is only about 7 days old, that is, almost as soon as the bacterial tissue is developed, and is complete by about the 15th day (figure 9, plate 14).

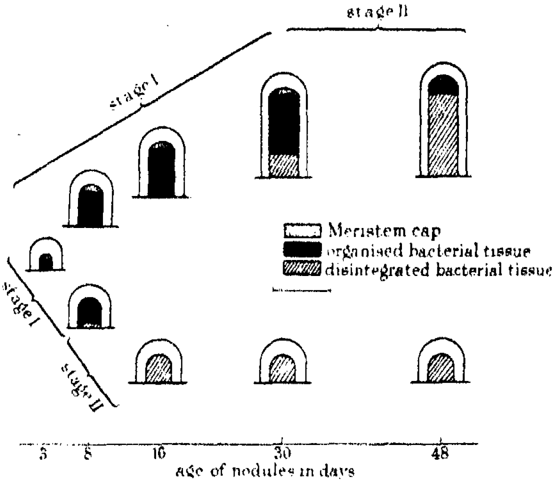

FIGURE 10. Growth and decay of the bacterial tissue in clover nodules.

Measurements of the volume of total bacterial tissue such as are plotted in figure 7, therefore give an incomplete picture of the differences between effective and ineffective nodules, for not only does the volume attained by the bacterial tissue differ in the two types of nodule, but also the duration of its active life prior to disintegration. It is the differences in volume of organized, or undisintegrated bacterial tissue throughout the life of the nodules that are of importance. These differences are illustrated diagrammatically in figure 10. The history of the bacterial tissue in a nodule can be divided into two stages. During stage I, which lasts from its first appearance until disintegration commences, the volume of the organized bacterial tissue is increasing. During stage II, the progress of disintegration from the base upwards causes a decrease in the volume of the organized bacterial tissue, which is in the end completely destroyed. In effective nodules the bacterial tissue continues to grow, so that on plants 10 weeks old its mean volume per nodule is 0·22 cu. mm. The nodule does not begin to show disintegration till it is nearly a month old and this process is not complete until it is about 8 weeks old, the organized bacterial tissue, formed by the end of the first week, having an active life of about 7 weeks. In ineffective nodules the bacterial tissues ceases to grow after about a week when its mean volume is about 0·05 cu. mm., and it is completely disintegrated by about the fifteenth day having thus an active life of little more than a week.

The cells of the bacterial tissue contain almost the entire bacterial population of the nodule, and this tissue, prior to its disintegration, may therefore be assumed to be the centre of nitrogen fixation. The activity of the nodule in fixing nitrogen must therefore be greatly affected by the differences above described, and should bear a relation to an integration of the volume of organized or undisintegrated bacterial tissue over the period covered by stages I and II.

D. RELATION OF VOLUME AND DURATION OF THE BACTERIAL TISSUE TO NITROGEN FIXATION IN CLOVER NODULES

A series of clover cultures in agar was made in order to obtain more exact data as to the changes in volume of organized and disintegrated bacterial tissue during nodule growth. The plants were grown in wide test tubes by the agar slope method above described. Half of the tubes were inoculated with the effective strain 2057 and half with the ineffective Coryn strain. The tubes were kept in a warm glasshouse and moisture maintained by the occasional addition of sterile culture solution.

Nodules were individually marked as they appeared, so that their age at the time of sampling might be known. Two tubes inoculated with each strain were taken at weekly intervals and the overall lengths of their nodules were recorded and measurements were made of the length and width of the organized and disintegrated bacterial tissue in these nodules, from free-hand sections, stained with 0·1 % thionin made up in a 5 % phenol solution. From these measurements the volumes of bacterial tissue were calculated, assuming a cylindrical shape. The results are shown graphically in figure 11, based on the examination of 152 nodules of strain 2057 and 71 Coryn nodules. If individual bacteria of the two strains are equally active in fixing nitrogen in the nodule, the amount of nitrogen fixed by a nodule should be proportionate to the product, vt, of the mean volume of organized bacterial tissue and the time during which it acts. This product was obtained from the data by calculating the term $\{S(x)/n\}\,t$, where x represents the volume of organized bacterial tissue found on n

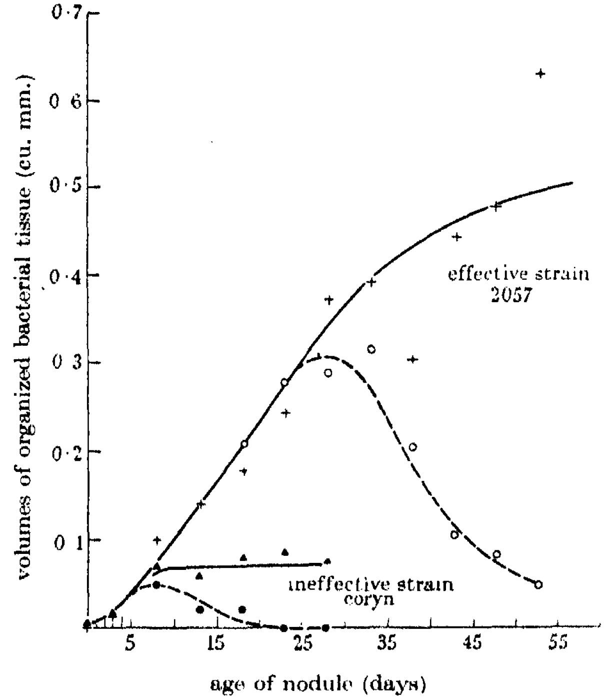

FIGURE 11. Changes in volume of total and of organized bacterial tissue in effective and ineffective clover nodules. ———— Total bacterial tissue; – – – – organized bacterial tissue.

occasions over a growth period of t days. The calculation expresses the result in units of 1 cu. mm. bacterial tissue acting for 1 day ('cu. mm.-days').

The value of vt was found to be 8·25 cu. mm.-days per nodule for the effective strain 2057 and 0·42 for the ineffective Coryn strain. The difference in the factor vt should therefore account for a difference of nearly twenty-fold in the nitrogen fixed by individual nodules of the two strains. To determine the true nitrogen fixing efficiency of the bacteria of each strain it was thus necessary to estimate the amount of nitrogen fixed per unit volume of the organized bacterial tissue per day of each strain.

An experiment was made to determine the quantities of nitrogen fixed by red clover, grown in quart milk bottles of sand as described in the section on methods. The bottles were divided into three sets, one uninoculated, one inoculated with strain 205 and one with the Coryn strain. After a growth period of about 3 months, the number of nodules in each bottle was counted and the nitrogen determined by the Kjeldahl method. The results are set out in table 1. The amount of nitrogen fixed per bottle was obtained by subtracting the amounts found in the uninoculated set. The amount fixed per ml., per day, e, was calculated from the formula $e = 1000f/vtd$, where f is the nitrogen fixed and d the mean number of nodules per bottle.* The value of e for strain 205 is 3·9 mg. of nitrogen fixed per c.c.-day, and that for the Coryn strain is 4·59. If one assumes that the latter strain has the same efficiency as strain 205, a calculation can be made of the nitrogen that should be fixed per bottle by the Coryn strain if the value of e for this strain were also 3·9. This calculation gives the expected amount of nitrogen fixed per bottle as 1·27 mg. which does not differ significantly from the observed value of 1·51 whose standard error is ± 0·547, with 11 degrees of freedom. Thus, although the plants with strain 205 nodules fixed nearly four times as much nitrogen as those bearing Coryn nodules, the whole of this difference can be accounted for by the smaller volume and shorter duration of the organized bacterial tissue in nodules of the ineffective strain.

TABLE 1. NITROGEN-FIXING EFFICIENCY OF CLOVER BACTERIA

EXP. I, 1936

	Uninoculated	Strain Coryn	Strain 205
No. of replicate bottles	12	12	16
Nitrogen per bottle (mg.)	0·91 ± 0·136	2·42 ± 0·53	6·80
Nitrogen fixed per bottle (mg.)	—	1·51 ± 0·547	5·89
Mean volume × duration of organized bacterial tissue per nodule in cu. mm. days (vt)		0·42	8·25
No. of nodules per bottle (d)		771	185
e = mg. nitrogen fixed per ml. active tissue per day = $1000f/vtd$		4·59	3·90

Applying $e = 3·90$ to data for Coryn.
Expected nitrogen fixed per bottle should be 1·27 mg.

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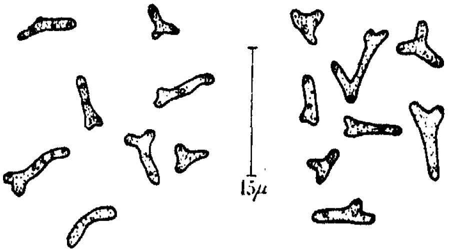
* In this experiment, the data used for calculating volumes of bacterial tissue were derived from agar cultures but applied to the analytical results obtained from sand cultures. This procedure seemed to be justified because the mean lengths of 205 and Coryn nodules were in approximately the same ratio in the agar and sand cultures.

E. THE STRUCTURE OF PEA NODULES PRODUCED BY EFFECTIVE AND INEFFECTIVE STRAINS

Observations were made on the anatomy of nodules produced by the effective strain 310 and by the ineffective strain B 33 on peas grown in small pots of sand. Roots were washed at different stages in the growth of the host plant, the nodules measured and sample nodules of various sizes were fixed and sectioned.

There was a marked difference in the size of nodules produced by the two strains. On plants 10 weeks old, strain 310 nodules of all ages had a mean length of 1·8 mm. and this included a number of long shaped and branched nodules. On plants of the same age, B 33 nodules had a mean length of 1·08 mm. and were spherical in shape.



ineffective strain, B 33 effective strain, 310

FIGURE 12. Bacteroid strains from pea nodules.

The course of nodule development in the pea generally resembles that in lucerne and clover. Sections showed that, on plants 10 weeks old, even large nodules of the 310 strain retained an active meristem cap and that very few showed any disintegration of the central tissue. Most of the nodules of B 33 strain, even on plants six weeks old, showed no active meristem cap and had their bacterial tissue completely disintegrated.

The appearance of the bacteria was particularly examined in view of the statements by Nobbe and Hiltner (1893) and Marie Löhnis (1930), that ineffective nodules contained bacteria in the rod stage which did not change into typical 'bacteroids'. In our material typical swollen and branched 'bacteroids' were found in the organized bacterial tissue of nodules produced by both the effective and ineffective strains (figure 12). These disappeared as usual during the process of disintegration of the bacterial tissue and so were absent in most nodules of the B 33 strain from plants over 6 weeks old, since by that time disintegration was usually complete.

Nobbe and Hiltner mention that their ineffective nodules showed no meristem cap and it therefore seems likely that they, at any rate, based their description on nodules in a state of disintegration, such as quickly follows the arrest of meristem activity. Marie Löhnis (1930) also describes certain unusual bacterial cells, referred to as 'brown bacteroids', in effective nodules. No such forms could be identified in our material, in which no constant differences in the shape of the bacterial cells could be found to distinguish the strains.

Young nodules produced by either strain contained considerable amounts of starch. Disintegrated bacterial tissue seldom contains any starch so that the earlier onset of disintegration caused an earlier disappearance of the starch in ineffective than in effective nodules.

Thus the principal differences found in effective and ineffective pea nodules are the smaller size of the latter and the shorter duration of the bacterial tissue in them. These differences are similar to those found in clover nodules.

F. THE COURSE OF GROWTH AND STRUCTURE OF SOY-BEAN NODULES PRODUCED BY EFFECTIVE AND INEFFECTIVE STRAINS

The material used for studying the anatomy of Soy bean nodules was obtained from sand cultures of the plants in large pots made in 1937, 1938 and 1939. The plants were inoculated with the effective strain 501 and with the ineffective strain 507, and nodules of various sizes were taken and fixed at different stages in the growth of the plant.

The development of nodules on the soy bean differs in some important respects from that in clover and pea nodules. Its general course has been described by Bieberdorf (1938) with whose account the present authors agree.

The nodules remain approximately spherical but become slightly flattened with age. Nodules of strain 501 continue to grow until they attain a considerable size. In the 1939 experiment, nodules of this strain, on plants four months old, attained a mean diameter, taken at right angles to the root, of 2·95 mm. Nodules of strain 507 stop growing quite early and remain small. The mean diameter of such nodules on four months old plants from the same experiment was only 1·9 mm. There is, in consequence, a large difference in volume of the bacterial tissue contained in the two types of nodule. Measurements were made of the bacterial tissue from freehand sections of 100 nodules of each strain taken from plants of varying ages grown in 1939. From these data the volumes were calculated, assuming the shape of the bacterial tissue to be an ellipsoid. The volume of bacterial tissue is plotted against the overall diameter of the nodule in figure 7. The relation of the two characters differs in the two strains, an effective nodule of given length having more bacterial tissue than an ineffective one. The volume of bacterial tissue, corresponding to the mean diameters given above are, 11·4 cu. mm. for strain 501 and 2·3 cu. mm. for strain 507.

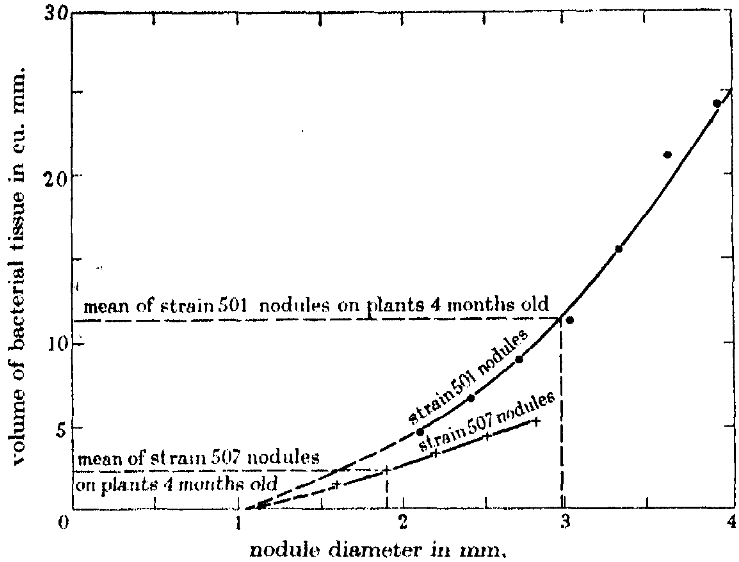


FIGURE 13. Relation of overall diameter to volume of bacterial tissue in soy-bean nodules. Each point gives the mean volume of bacterial tissue in from three to thirty-two nodules of the same overall length.

The bacterial tissue of soy-bean nodules differs in appearance from that of clover and pea nodules. In the latter the great majority of cells are filled with bacteria both in effective and ineffective nodules, so that the volumes of this tissue in the two types gives a comparable estimate of the relative numbers of bacteria present. But in soy-bean nodules the bacterial tissue contains a relatively large proportion of uninfected cells and this proportion differs strikingly in effective and ineffective nodules studied (figures 14, 15, plate 14). Estimates of the percentage volumes of infected cells in the bacterial tissue were obtained by measuring the areas occupied by infected and by uninfected cells in random microscope fields in microtome sections from a number of nodules of each type. The results are shown in table 2. Measurements made from soy-bean nodules grown in 1938 showed that 82·99 % of the volume was occupied by infected cells in strain 501 nodules and 43·44% in strain 507 nodules. In the material grown in 1939, the percentages were 94·19 for strain 501 and 49·16 for strain 507. The percentages for the two strains in each year were in the same ratio, namely 1·91 : 1, but they were different in the two seasons, this seasonal difference being significant in the case of strain 501. In the infected cells of both types of nodules, the bacteria lie close-packed in the cytoplasm, so that a comparison of the percentage volumes of infected cells should provide an estimate of the relative numbers of bacteria per unit mass of the bacterial tissue in the two types of nodule.

Table 2

	501 nodules		507 nodules	
	1938	1939	1938	1939
No. of nodules examined	5	11	5	11
Percentage volume of infected cells (mean)	82·99	94·19	43·44	49·16
Standard error	± 3·15	± 1·05	± 5·19	± 2·05

In soy-bean nodules the infected cells were found to be free from starch, but the uninfected cells contained many starch grains. Since these latter cells were much the more numerous in inefficient nodules, the amount of starch in these nodules was proportionately greater. The 507 nodules somewhat resembled the ineffective *Phaseolus* nodules, described and illustrated by Elizabeth McCoy (1929), in having many uninfected cells filled with starch in the central tissue.

The bacteria in infected cells in soy-bean nodules remain in the rod stage and show scarcely any change toward the 'bacteroid' condition in any part of the nodule. There was no noticeable difference in their appearance in the organized bacterial tissue in nodules produced by the two strains.

✓The process of disintegration of the bacterial tissue in soy beans differs in important respects from that in clover and pea nodules. It does not always begin at the base but may start at any point in the central tissue. In the early stages, the bacteria break up into granules and the infected host cells lose their turgidity. The uninfected cells, however, remain turgid and their pressure on the infected cells causes these to collapse. When this process is far advanced the appearance of a section suggests, and was, indeed, at first mistaken for intercellular infection (figure 16, plate 14). It probably accounts for the statement of Kâs (1930) that soy-bean nodules show both intra- and intercellular infection, and may also explain the 'intercellular' type of infection described in *Serradella* by Milovidov

(1926, 1928). Bacterial tissue showing this collapse of the infected cells is here referred to for convenience as 'disintegrated', although there is no general collapse of the tissue as occurs in clover and pea nodules. This is clearly due to the large proportion of uninfected cells which remain turgid, and support the tissue.

There is a marked difference between nodules of strains 501 and 507 in the age at which disintegration takes place. In the 1939 nodules of strain 507 the process began when the host plant was only 4 weeks old and was nearly complete by the twelfth week. Strain 501 nodules on plants twelve weeks old did not yet show any disintegration, which only began to appear when the plants were at the end of their growth period, at an age of about 17 weeks.

Effective and ineffective soy-bean nodules thus show differences in their growth and decay of the same general type as those found in clover and pea nodules. Effective nodules grow to a mean diameter 50 % greater than that of ineffective nodules. They contain more than five times the volume of bacterial tissue, which bears twice the percentage by volume of infected cells, and this tissue lasts more than four times as long as in ineffective nodules before becoming disintegrated.

It was, however, necessary to follow the course of growth and decay of the bacterial tissue throughout the growing period of the plant before the quantitative effect of these factors on nitrogen fixation could be estimated.

The size of the plant made it impracticable to use the agar culture method, used with clover for obtaining individual nodules of known age. The measurements here recorded are therefore the means from batches of nodules taken from successive reapings of plants of recorded ages taken during the pot-culture experiment of 1939. These data did in fact give a good measure of the growth and decay of the nodules because, as is usual in soy beans, nearly all the nodules appeared when the plants were quite young and so did not vary greatly in age within each batch.

The soy beans were grown in thirty large pots, twelve of which were inoculated with strain 501, twelve with strain 507, while six were left uninoculated. Three or four plants inoculated with each strain were removed at weekly intervals, and, from these, twenty nodules of each type were examined by means of hand sections stained with carbol thionin. In each section, measurements were made of the diameters of the nodules and of the area occupied by bacterial tissue, and an estimate was made of the percentage of that area showing disintegration. From these measurements, the mean volumes of organized and of disintegrated bacterial tissue in each batch of twenty nodules were calculated. The volumes of total (that is, organized plus disintegrated), bacterial tissue have been plotted against nodule diameter in figure 13, already discussed. Changes in volume of the organized bacterial tissue, with time are shown in figure 7, and discussed below. At the end of the growth period, the remaining plants, then 4 months old, were reaped, the nodules counted and nitrogen determinations made by the Kjeldahl method.

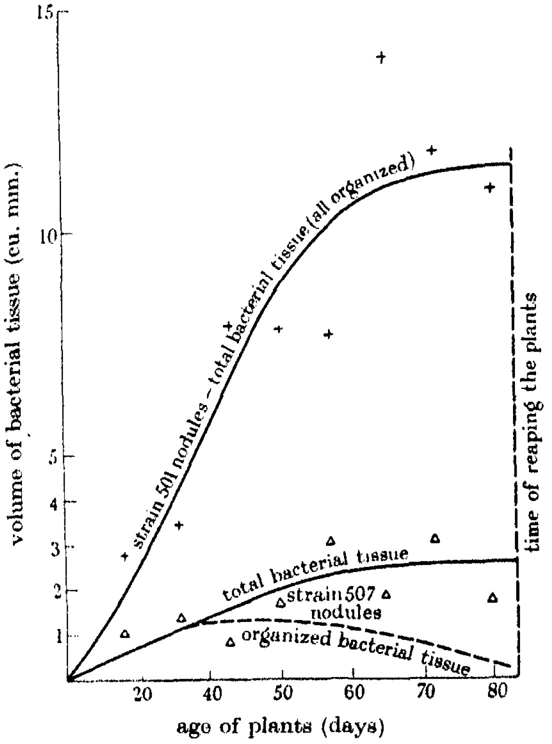


Figure 17. Changes in volume of organized and total bacterial tissue in effective and ineffective soy bean nodules. Each point represents the mean of 20 nodules.

In figure 17, the calculated volumes of total and of organized bacterial tissues, taken from the weekly measurements, are plotted against the age of the plants. From these data the integrated cu. mm.-days, *vt*, are calculated according to the formula

$$vt = \frac{S(b-by)}{n100}t,$$

where b is the mean volume of total bacterial tissue found on each occasion and y the estimated percentage of that tissue showing disintegration.

The values of vt thus calculated, are 83·95 for strain 507 and 647·4 for strain 501. These are in units of 1 cu. mm. of *organized* bacterial tissue acting for one day, but they do not represent the relative volumes of *active* tissue until further corrected for the percentage, c, of infected cells within the bacterial tissue of each strain. When this correction is made, using the mean percentages obtained from 1939 data shown in table 2, the value of $vtc/100$ for strain 507 is 41·27 and for strain 501 it is 609·79. The average 501 nodule should thus fix nearly fifteen times as much nitrogen as the average nodule of strain 507, by reason of its larger content of infected cells.

The data with regard to nitrogen fixation are set out in table 3. Plants with 501 nodules fixed about six times as much nitrogen as did those with 507 nodules. The quantity e of nitrogen fixed per ml. of active infected cells per day was calculated from the formula $e = 1000f\Big/vt\frac{c}{100}d$ where f and d are means per plant for nitrogen fixed and nodule numbers respectively.

TABLE 3. NITROGEN-FIXING EFFICIENCY OF SOY-BEAN BACTERIA (1939 EXPERIMENT)

	Uninoculated	Strain 507	Strain 501
No. of plants	40	33	18
Nitrogen per plant (mg.)	16·69	19·34	31·70
Nitrogen fixed per plant (mg.)		2·65	15·01
Mean volume × duration of organized bacterial tissue per nodule in cu.mm.-days (vt)		83·95	647·4
Infected cells as percentage of organized bacterial tissue (c)		49·16	94·19
No. of nodules per plant (d)		45·2	21·1
e = mg. nitrogen fixed per c.c. active infected cells per day $= 1000f\Big/vt\frac{c}{100}d$		1·42	1·17

Applying $e = 1·17$ to data of strain 507.
Expected nitrogen fixed per plant should be 2·18.

The value of e, for the two strains 507 and 501, were 1·42 and 1·17 respectively. If the value for strain 507 were the same as for the other strain, namely 1·17, the expected nitrogen fixed per plant would be 2·18 mg., actually a fraction less than the observed figure. Thus the whole of the large difference in nitrogen fixed by soy-bean plants bearing the two types of nodules can be accounted for by differences in the total volume of infected cells and in the duration of their activity.

DISCUSSION

The course of the growth and decay of nodules produced by effective and ineffective strains of bacteria on clover, peas and soy beans thus shows that there are two quantitative characters closely correlated with effectiveness, namely the combined volume of the infected cells and the length of time that elapses before they collapse and disintegrate. Other features of nodule anatomy, unless causally connected with the above two characters, were found to show no association with effectiveness. Attempts were made with clover and soy beans to give quantitative values to these factors of volume and duration of the organized tissue containing bacteria, and to apply these values as corrections in estimating, from the results of analysis, the quantities of nitrogen fixed by a unit volume of infected cells in unit time. These attempts were so far successful that in experiments with both host plants, the whole of the large differences between the amounts of nitrogen fixed by 'effective' and 'ineffective' strains respectively could be accounted for by the differences in volume and duration of the infected nodule cells. The data showed no evidence that the 'ineffective' strains were really less efficient in fixing nitrogen per unit of bacterial mass in unit time, than the 'effective' strains.

The problem of ineffective nodules thus resolves itself into the need for explaining why the volume of infected cells in them remains so small and why these cells disintegrate so soon. The small volume of bacterial tissue is related to the early cessation of apical meristem growth in the nodule, but since this meristem activity is doubtless stimulated by the bacteria, both the short growth period of the nodule and the early decay of the infected cells are indications of poor bacterial growth within the tissues.

Strains of nodule bacteria differ in the rapidity of their growth on artificial media, but there is no correlation between this character and their effectiveness in the host plant. There is thus no reason to believe that ineffective strains are naturally less vigorous in growth than others, when given a suitable medium. Hence the poor growth of these strains within the nodule indicates that the tissues of the host plant provide an environment that is less suited to the ineffective than to the effective strains.

This may either mean that some unfavourable factor is normally present in the host tissues, or that such a factor appears in them as a consequence of infection by the ineffective strain. The latter alternative is rather suggested by the fact that ineffective nodules commence their growth quite normally and only later show arrested development.

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Description of plates

Plate 13

Figures 1 to 3. Development of an ineffective clover nodule produced by the Coryn strain.

Figure 1. Nodule 1 day old, composed of meristem tissue.

Figure 2. Nodule 3 days old, showing swelling of the central tissue cells, and formation of a vascular strand.

Figure 3. Nodule 6 days old, and about fully grown, showing the small amount of bacterial tissue.

Figure 5. Effective clover nodule one month old produced by strain 205, showing large development of bacterial tissue.

Figure 8. Six weeks old nodule produced by strain 205. Disintegration of the bacterial tissue commencing at the base.

m.c. meristem cap; *o.b.t.* organized bacterial tissue; *d.b.t.* disintegrated bacterial tissue; *v.s.* vascular strands; *r.s.* stele of root.

Plate 14

Figure 9. Clover nodule, 15 days old, produced by the Coryn strain, showing disintegration of the bacterial tissue. *d.b.t.* disintegrated bacterial tissue; *v.s.* vascular strands; *r.s.* stele of root.

Figures 14 to 16. Sections of the bacterial tissue of soy-bean nodules.

Figure 14. Strain 501 (effective), showing large proportion of infected cells, *i.c.*

Figure 15. Strain 507 (ineffective), showing small numbers of infected cells and large proportion of uninfected cells, *s.c.* containing starch.

Figure 16. Bacterial tissue of strain 507 nodule undergoing disintegration through collapse of the infected cells, *i.c.*, the sterile cells, *s.c.*, remaining turgid.

Plant pigments in the Galápagos Islands

By T. W. J. Taylor

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The Galápagos Islands were visited by the Lack-Venables Expedition during the winter 1938–9, and analyses were made of the anthocyanin pigments contained in the characteristic and largely endemic plants of Indefatigable Island. The methods used for anthocyanin identification under camp conditions are described. No new pigments were found, but a high proportion of the plants were found to contain nitrogenous anthocyanins. This provides powerful support for the views already put forward as to the distribution of such pigments (Lawrence, Price, G. M. Robinson and R. Robinson 1939), although slight modification of these views is needed.

During the winter 1938–9 the Lack-Venables Expedition worked on biological problems in the islands of the Galápagos Archipelago and a stay of two months (February and March) on one of the larger islands, Indefatigable, afforded the opportunity for systematic botanical collection. This was combined with the study on the spot of the types of pigments found in the plants; the present paper deals with those of the anthocyanin group. The purpose of this study was to see how far identification of anthocyanin pigments can be carried out in the field with the restrictions in apparatus and reagents which transport to such a part of the world and work under camp conditions necessitates, and to examine the pigments present in as many as possible of the species which are entirely confined to these islands.

The Galápagos Islands are a volcanic group and lie on the equator in the Pacific about 600 miles west of Ecuador. They have long been recognized as remarkable for their fauna, in which reptiles predominate and mammals are hardly represented, and for their flora, of which over 40 % of the species are said to be endemic. In the larger islands, which rise in volcanic peaks to over 2000 ft., there is a marked succession of botanical zones, from the very dry regions near the coast through dense forest at intermediate heights to a fern belt and finally open country of a moorland type, and consequently species of varied character are found. In the drier regions many of the plants spend much of the year in a resting phase, but the investigation was carried out at the end of the short rainy season when all the plants were in vigorous growth.

The methods employed for the identifications of the anthocyanins were those developed by R. Robinson and G. M. Robinson and already used by them for a very large number of plants (1931, 1932, 1933, 1934; see also Lawrence, Price, G. M. Robinson and R. Robinson 1938). Certain simplifications had to be introduced because of the impossibility of transporting large quantities of organic solvents and of working with them in a tropical climate. When there is only one anthocyanin present, the colour reactions with simple reagents and the partition between amyl alcohol and aqueous hydrochloric acid together with the colour reactions and distributions of the anthocyanidin are sufficient to establish its nature, but when the pigment is a mixture of anthocyanins, separation of them involves larger quantities of material and much larger quantities of solvents, so that complete identification of the constituents becomes impracticable in the field; in such cases the results given below are provisional and are based to some extent on personal judgement and experience. One of the most valuable reagents taken was ethyl acetate, which, on Lady Robinson's suggestion, was used extensively for removing yellow and brown anthoxanthins from acid solutions of anthocyanidins and thus made possible many of the identifications; since it is also admirable in entomological killing bottles, it is a useful dual purpose reagent. The apparatus taken was a small glass mortar for crushing plant material, test-tubes and two small separating funnels. Organic solvents such as amyl alcohol, benzene and petroleum ether were taken in small square tins, and quantities of 350–400 g. proved amply sufficient, apart from petroleum ether; this is needed in larger amount for returning into solution in aqueous hydrochloric acid anthocyanidins which have been extracted with amyl alcohol. The supply taken was soon exhausted, but it was found that petrol for a motor-boat could be used in its place. The most difficult reagent to transport under primitive conditions is concentrated hydrochloric acid, which is essential for the hydrolysis of an anthocyanin, and this was packed in a separate wooden box. Experiments had been made before leaving England with the view of finding some solid acid sufficiently strong for the purpose, but they were unsuccessful; potassium hydrogen sulphate can be used as a dilute mineral acid, but even in saturated solution it does not hydrolyse an anthocyanin at the requisite speed. A supply of rain water was available for the analyses, and 'meta' fuel was used for heating test-tubes.

The results obtained are given below. The identifications of the plant species are to some extent provisional; specimens of all the species were brought home and are now in the Herbarium of the Royal Botanic Gardens, Kew, but the difficulties of the present international situation have made the systematic working out of the collection impossible. The majority of the plants in Galápagos do not produce conspicuous flowers, a fact which has been attributed to the paucity of insect life in the islands (Robinson 1911), and several of those which do, have yellow flowers, containing no anthocyanin; however, anthocyanins can readily be found in the stems or leaves of several of the yellow and white flowered species.

The most striking feature of the results is the high proportion of species in which the anthocyanin is not of the normal type (i.e. a derivative of pelargonidin, cyanidin or delphinidin), but a nitrogenous anthocyanin of the kind that is found in the beetroot (Robinson and Ainley 1937) and in the bracts of *Bougainvillea* (Price and Robinson 1937); more than one-third of the dicotyledons examined contained such pigments. The nitrogenous anthocyanins are readily detected by their colour reactions, but, in the field at least, no distinction can be drawn between them, and they are simply described below as nitrogenous. It was soon realized that the reason for their high frequency is that certain plant families contribute a larger proportion of species to the Galápagos flora than they do to the European floras, and that members of these families invariably contain nitrogenous, and not ordinary, anthocyanins. This latter fact has already been pointed out by Lawrence, Price, G. M. Robinson and R. Robinson (1939), and the truth of their generalization, which was based on the plants native to or introduced into Europe, finds a striking confirmation in the Galápagos observations, which were made independently and without a knowledge of the generalization. Several interesting examples of how a plant belonging to these certain families seems to be incapable of producing any but a nitrogenous anthocyanin were found; thus *Cereus Thouarsii* Weber, an endemic tree cactus, has a waxy flower pale mauve on the outer side and its fruits are pale maroon; *Sesuvium Portulacastrum* L., a shore succulent, has pink flowers and the old stems exposed to the sun become bright lobster red; a herbaceous plant of the forest, *Boerhaavia scandens* L., has small pink flowers and as the plant grows old, the nodes on the larger stems become dull red; in all these cases the pigment is nitrogenous.

Lawrence, Price, G. M. Robinson and R. Robinson have further pointed out that the occurrence of nitrogenous anthocyanins only in certain families may be of importance in tracing the relationships between the groups of plants, and that the results so far obtained fit well with the scheme of systematic relationships most recently proposed (Hutchinson 1926) with one exception, the order Cactales. All the other orders in which these pigments have been detected are shown in Hutchinson's scheme as closely related, but Cactales are placed far away from the others, although Hutchinson states that there are grounds for placing them next to Ficoidaceae. Since the latter family invariably contains nitrogenous pigments, the above authors point out that from a chemical point of view this alternative position is to be preferred.

There is, however, another clash between the morphological and chemical deductions. In their collected results the above authors included an observation of Kryz (1920) that the pigment of *Fuchsia* berries is nitrogenous, and hence placed the order Lythrales among those with such pigments; this would agree with Hutchinson's scheme, in which Lythrales are shown as deriving from Caryophyllales and leading on to Thymelaeales. At least two of the families of the order Caryophyllales (Ficoidaceae and Portulacaceae) and the only family of Thymalaeales so far examined (Nyctaginaceae) invariably contain nitrogenous pigments. Kryz's result is, however, erroneous. Dr J. R. Price has re-examined certain *Fuchsia* berries and finds only normal anthocyanin, and no other member of the order to which *Fuchsia* belongs (Lythrales) has been found to contain a nitrogenous anthocyanin. There are several instances already published (see Lawrence, Price, Robinson and Robinson 1939) and others are given here, including some English plants investigated for this purpose since my return to England. Hence the present position is that the order Lythrales, placed by Hutchinson as leading from an order in which nitrogenous anthocyanins occur to another in which, as far as is known, they are invariable, itself contains no such pigment. Although it is not known how strong such evidence is, from a chemical point of view Hutchinson's sequence is unsatisfactory.

In the following list of results the plants are arranged in Hutchinson's classification and the numbers shown are those allotted by him to the orders and families; the arrangement is reversed so that the more advanced types are given first. Species which are endemic in the Galápagos Archipelago are marked with an asterisk. The nitrogenous anthocyanins were recognized by the immediate yellow colour formed on adding sodium hydroxide to the acid solution, the destruction of the pigment by ferric chloride, the ease of oxidation in alkaline solution and the characteristic dilute permanganate colour of the acid solution.

METACHLAMYDEAE

76 Lamiales

263 VERBENACEAE. **Clerodendron molle* HBK. var. *glabescens* Svensen. The white corolla has a roseate tube; the pigment contains much anthoxanthin, but also a monoglucoside of an anthocyanidin which is almost certainly peonidin.

75 **Personales**

259 Acanthaceae. *Elytraria squamosa* (Jacq.) Lindau. The minute blue corolla contains delphinidin diglucoside.

**Justicia galapagana* Lindau. The purple flowers contain peonin.

74 **Solanales**

251 Convolvulaceae. *Evolvulus hirsutus* HBK. The small blue flowers contain a diglucoside of malvidin.

Ipomaea batatas Poir. The red stems of the sweet potato contain much anthoxanthin and a diglucoside of pelargonidin with possibly a little cyanidin.

I. pes-caprae Sweet. The flowers contain cyanidin monoglucoside.

I. triloba L. The flowers contain peonidin diglucoside with possibly some cyanidin.

Calonyction aculeatum (L.) House. The stems of this climber are in parts red and contain much anthoxanthin and some cyanidin monoglucoside.

250 Solanaceae. **Cacabus Miersii* (Hook.) Wettst. The only parts of this shore plant which contain anthocyanin are the petioles of the young leaves and the flower in bud (when open it is white); the pigment is malvidin diglucoside.

73 **Boraginales**

249 Boraginaceae. **Coldenia Darwinii* (Hook.) Gürke. The backs of the leaves at the top of the shoots are purple and contain a monoglucoside of cyanidin.

ARCHICHLAMYDEAE

41 **Leguminosae**

148 Papilionaceae. *Erythrina velutina* Willd. The scarlet flowers of this tree contain a pelargonidin diglucoside.

Galactea Jussiaeana Kunth, var. *volubilis* Benth. The small pink flowers contain a malvidin diglucoside.

147 Mimosaceae. *Acacia macracantha* H. and B. In the seed-pod before it is ripe there is a thin pulpy layer, as of red jam, beneath the outer skin; this is very rich in a monoglucoside of cyanidin, together with an anthoxanthin which gives a solution in ethyl acetate much redder than most.

38 Euphorbiales

136 EUPHORBIACEAE. **Euphorbia recurva* Hook. The stems are purple and the leaves purple green; they contain a mixture of anthocyanins, diglucosides, probably of delphinidin and cyanidin.

Manihot utilissima Pohl. The red stems of this cultivated plant contain a diglucoside of peonidin.

36 Malvales

132 MALVACEAE. **Gossypium Darwinii* Watt. The faded leaves are deep red and contain a monoglucoside of cyanidin, possibly with other anthocyanins as well.

Hibiscus rosa-sinensis L. The cultivated scarlet hibiscus contains a cyanidin diglucoside mixed, probably, with a peonidin derivative.

H. tiliaecus L. The faded flowers are a rich red-brown, but no anthocyanin could be detected.

33 Myrtales

120 MELASTOMACEAE. **Miconia Robinsoniana* Cognieux. The minute purple flowers contain an anthocyanin, but the colour reactions are masked by the presence of some colourless compound which gives an intense blue colour with ferric salts. Unfortunately the flowers are very small and the shrub grows only in the more inaccessible parts of the island.

31 Cactales

107 CACTACEAE. **Cereus Thouarsii* Weber. The outer part of the flowers and the fruit contain a nitrogenous anthocyanin.

22 Thymelaeales

83 NYCTAGINACEAE. *Boerhaavia coccinea* Mill. The small purple flowers contain a nitrogenous anthocyanin.

B. scandens L. A pigment of the same type is in the pink flowers and the red nodes of the older stems.

Cryptocarpus pyriformis HBK. The old hardened shoots of this vine are faintly red in places and the anthocyanin is nitrogenous.

21 Lythrales

72 LYTHRACEAE. *Cuphea strigulosa* H.B.K. The bright purple flowers contain a diglucoside of cyanidin.

19 **Chenopodiales**

All the species in this order contain a nitrogenous anthocyanin in the parts given.

63 AMARANTACEAE. *Alternanthera ficoidea* (L.) R.Br. Stems and leaves.

**A. hamilifolia* (Lam.) Standley, *macrophylla* Howell. Stems and leaves.

Amaranthus gracilis Desf. Stems and leaves, especially the outer layer of the stem.

A. quitensis HBK. Stems.

Celosia argentea L. Flower.

59 PHYTOLACCACEAE. *Phytolacca australis* Phil. Flower and flowering shoot.

17 **Caryophyllales**

56 PORTULACACEAE. *Portulaca oleracea* L. The red stems are rich in a nitrogenous anthocyanin.

55 FICOIDACEAE. *Sesuvium Portulacastrum* L. The pink flower and the lobster red stem contain a nitrogenous anthocyanin.

7 **Piperales**

28 PIPERACEAE.

**Peperomia petiolata* Hook.

**P. galapagensis* Hook.

In some plants of both species, particularly those growing in the sun, the thick fleshy leaf has a very narrow purple edge; there is also a purple colour in the stems of the young plants. The pigment contains so much anthoxanthin that the anthocyanin reactions are obscured, but after hydrolysis and repeated washing with ethyl acetate, cyanidin can be detected with certainty, and since the original pigment is insoluble in amyl alcohol, it must be present as a diglucoside.

MONOCOTYLEDONS

ORCHIDACEAE. *Ionopsis utricularioides* (Sw.) Lindl. The very pale mauve flowers of this epiphytic orchid contain a delphidin diglucoside together with another, probably cyanin.

GRAMINEAE. *Paspalum conjugatum* Berg. The damaged leaves of this grass become purple-brown and contain an anthocyanin and much anthoxanthin which makes identification of the former difficult. Hydrolysis, however, shows that the anthocyanin is derived from cyanidin.

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Adiantum macrophyllum Sw. The young fronds of this fern are translucent and bright pink; they contain much anthoxanthin and also an anthocyanin based on cyanidin. No anthocyanin can be detected in any part of the mature plant. Anthocyanins have been found in other young ferns, some derived from cyanidin and others from anthocyanidins of an unknown type (Price, Sturges, R. Robinson and G. M. Robinson, *Nature, Lond.*, 1938, **142**, 356).

The following British plants (dicotyledons) have been examined.

Family	Species	Part of plant	Anthocyanin
Onagraceae	*Epilobium angustifolium* L.	Young shoot	Cyanidin monoglucoside with probably some malvidin monoglucoside
	E. hirsutum L.	Red stems	Peonidin monoglucoside
Polygonaceae	*Rumex conglomeratus* L.	Red stems	Peonidin monoglucoside
	R. crispus L.	Red stems	Much anthoxanthin and peonidin pentosoglucoside
		Damaged leaves	Cyanidin monoglucoside
Halorrhagaceae	*Hippuris vulgaris* L.	Leaves and stems	Cyanidin monoglucoside
Ceratophyllaceae	*Ceratophyllum submersum* L.	Leaves	Malvidin monoglucoside

My warmest thanks are due to Mr J. P. M. Brenan and to Mr N. Y. Sandwith of the Royal Botanic Gardens for their identifications of the Galápagos material, to my wife for her invaluable help in the field, to the inhabitants of the Indefatigable Island for their help and hospitality, and to Sir Robert and Lady Robinson and Dr J. R. Price for their advice and helpful discussion.

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The reduction of ferric oxalate by isolated chloroplasts

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1. The isolated chloroplasts from *Stellaria media* show a progressive fall in activity approaching zero in 3–6 hr. Four different strains of the plant were grown which showed differences in the stability of chloroplasts after removal.

2. Two methods have been used to measure the activity of chloroplasts:

(*a*) The measurement with HbO_2 of oxygen produced from ferric potassium oxalate as previously described.

(*b*) The measurement of the rate of reduction of methaemoglobin in presence of atmospheric oxygen, the methaemoglobin being reduced by the ferrous iron.

3. The Q_{O_2}, measured as rate of oxygen production calculated on the basis of dry weight of leaf taken, is about 20. The Q_{O_2}, measured as rate of methaemoglobin reduction, generally appeared less as the reduction of methaemoglobin by ferrous iron is relatively slow.

4. The reduction of methaemoglobin in presence of ferric potassium oxalate has been studied quantitatively from the point of view of iron, methaemoglobin, and chloroplast concentration.

5. The effect of different light intensities on the ferric oxalate reaction is similar to the effect of varying light intensity on photosynthesis in whole plants and lies within the range of values found by different workers.

6. The ferric oxalate reaction is inhibited by urethane. Phenyl urethane inhibits in much smaller concentrations than ethyl urethane. The effective concentrations of urethane are similar to those affecting photosynthesis.

7. It is concluded from the present observations that the light reaction in vegetable photosynthesis is the production of the oxygen molecule and is not the reduction of carbon dioxide.

1. INTRODUCTION

The photosynthesis of green plants is characterized by the production of molecular oxygen when the living cells containing chlorophyll are illuminated. The oxygen, however, is only produced when carbon dioxide is taken up, and no other substance at present known will cause the photosynthetic reaction on the living cell. The chloroplasts, which are organized subcellular units containing chlorophyll, will themselves evolve traces of oxygen in light after removal from the cells. It was shown (Hill 1939) that, in light, chloroplasts would reduce ferric oxalate to ferrous oxalate and that an equivalent amount of oxygen was set free. This reaction, here referred to as 'the ferric oxalate reaction', was capable of measurement and is probably the first indication of any measurable activity apart from the whole cell which has any bearing on photosynthesis. The chloroplasts, after removal from cells, have this property of evolving oxygen from certain hydrogen acceptors, but carbon dioxide will not act as a hydrogen acceptor. Moreover, the oxygen produced by isolated chloroplasts is maintained only at a very low pressure of 2–4 mm. Hg; a possible explanation of the low pressure of oxygen is the re-oxidation of some hydrogen acceptor. In spite of this, however, with the help of the respiratory pigment, haemoglobin, it is shown that the chloroplasts over this low range of oxygen pressure have a high activity when compared with other biological systems. The fact that the chloroplasts will produce molecular oxygen from substances other than carbon dioxide, gives a possibility of analysing the photosynthetic mechanism from a more chemical point of view. Hence it is of primary importance to establish, by varying external and internal factors, the behaviour of the chloroplast outside the living cell. The present paper is concerned with the effect of varying light intensity and the inhibiting effect of urethane on the ferric oxalate reaction. In a previous investigation it was found that cyanide and hydroxylamine had very little effect on the ferric oxalate reaction of chloroplasts, yet they are known to affect photosynthesis. The object of the present investigation is to extend the comparison of the ferric oxalate reaction with the present knowledge of photosynthesis.

The oxygen output has been determined by using haemoglobin and the reduction of iron by using methaemoglobin. The activity, or rate of reaction observed, is expressed as a positive Q_{O_2} calculated from the dry weight of the actual leaves used for the preparation of the chloroplast suspensions; this refers all the data to the original plant tissue.

2. METHOD AND MATERIALS

The experiments were carried out in tubes of 1·5 cm. diameter. The chloroplasts were used in a dilution so that the total absorption of light was small. The illuminating device consisted of a projection lamp (Mazda projector lamp Class A1, 200 V, 250 W) with a lens system and trough of water as follows. Immediately beneath the spectroscope are the cups of the spectrocolorimeter described by Hill (1936):

0	Projector lamp.
5·1 cm.	Condensing lens, focal length 5·1 cm., diameter 4·7 cm.
10·1 cm.	Water trough, for cooling, thickness 5·1 cm.
16·2 cm.	Lens, focal length 20·3 cm., diameter 6 cm.
17·2 cm.	Ground-glass screen.
19·4 cm.	Water trough for containing experimental tubes, thickness 3·0 cm.
23·9 cm.	Aperture of Zeiss microspectroscope.

The method of estimating the intensity of illumination was as follows:

The illumination of the lamp used in foot-candles was known (supplied by makers) and the light transmitted by our system of illumination was compared with the original source. The comparisons were made with the times taken for a P.O.P. paper (Paget) to reach a series of standard tints. This method is only reliable when the times of exposure for the standard and the unknown to reach the same tint are nearly equal, that is, only nearly equal intensities of illumination can be directly compared. The standard tints, which were washes of colour on paper, were calibrated by exposing the paper to known intensities of illumination until a match was obtained, when the time was recorded. The tints, six in number, were arranged in order as strips with equal spaces between them (3 mm.); the P.O.P. was put behind and the times were read for the paper to match all six tints in turn. This allows six readings to be taken for each illumination. This method does not depend on the sensitivity of the paper being strictly linear, but it was found that a linear relation held to allow variations in the time of exposure of about 50%. This method of measuring the light intensity gives values which can be compared with data by other workers on photosynthesis where gas-filled lamps were used. The intensity of illumination was varied by using screens of oxidized copper gauze mounted between glass which were interposed between the ground-glass screen and the experimental vessel. The screens were calibrated on the Nutting photometer. The light intensities in lux were as follows:

500 W lamp	40,300 lux
250 W lamp	18,600 lux
250 W lamp and 1 screen	6,200 lux
250 W lamp and 2 screens	2,070 lux

Unless otherwise stated all experiments were carried out with an illumination of 18,600 lux.

The activity of the chloroplasts from *Stellaria media* shows considerable variation. In order to obtain repeatable results a standard method was used to obtain chloroplasts from this plant. Leafy shoots from plants, shortly before flowering, were picked at 10 a.m., the stems were placed in water for ½ hr. and then the young fully grown leaves removed and soaked in tap water for at least ½ hr.; samples could then be taken out during the day until 6 p.m. If the leafy shoots were picked later in the day, the activity was always less, falling nearly to zero if a specimen was taken during a sunny afternoon. The chloroplast suspensions were prepared by grinding 0·6 g. fresh leaves of *S. media* previously soaked in water with 3·0 c.c. of 12 % sucrose buffer pH 7·9 containing M/30 phosphate. The fluid was filtered through a pad of glass-wool to remove epidermis and broken tissue. This suspension was then equivalent to 0·06 g. dry weight of leaf in 3·5 c.c. fluid and was about $4 \cdot 4 \times 10^{-4}$ M with respect to chlorophyll. The molecular absorption coefficient $\log_{10} (I_0/I)$ for chlorophyll in acetone was taken as $4 \cdot 6 \times 10^4$. The results of the experiments are given in terms of Q_{O_2} calculated on 0·6 g. of leaf taken in a volume of 3·5 c.c.

There are two methods of determining the activity of chloroplasts in reducing ferric oxalate under various conditions. The first method depends on the rate of oxygen production and has been described previously (Hill 1937, 1939). The second method is to measure the rate of production of ferrous iron and will be described in § 4.

3. MEASUREMENT OF OXYGEN PRODUCTION BY CHLOROPLASTS

The muscle haemoglobin was prepared from shin beef as described previously (Hill 1939).

The iron solution consisted of 0·96 g. ferric ammonium alum dissolved in 1000 c.c. of N/100 HCl. This solution was found to keep well. The potassium oxalate was M/2. The mixture of ferric potassium oxalate and haemoglobin was made up as follows. To 1 c.c. of potassium oxalate the required amount of the iron solution was added and the excess of acid neutralized with NaOH. 0·5 c.c. of M/7·5 phosphate buffer pH 7·9 was added and sufficient haemoglobin added to bring the concentration in the region of $0 \cdot 7 \times 10^{-4}$ g. atoms haemoglobin Fe per litre when the volume was made up to 5 c.c.

This mixture was placed in a vacuum tube 1·5 cm. in diameter and evacuated with shaking until all the oxygen had been removed. 0·4 c.c. of a chloroplast suspension was now added down the side tube and the tube evacuated again with cautious shaking. The tube was placed in the trough and illuminated, and the oxygen evolved was followed in the colorimeter, readings being taken at convenient intervals with a stopwatch.

The readings of a typical experiment are shown in figure 1; the rate of oxygen production is plotted for four different light intensities. It will be noticed that the results lie on a curve. For determining the rate of oxygen production, the initial slope of the curve is always used. Where this is not sufficiently pronounced the convention is adopted of taking the slope represented by the readings from the curve at 0 and 100 sec. The Q_{O_2} is arrived at in the following way. A solution of haemoglobin containing $1\cdot0 \times 10^{-4}$ g. atoms haemoglobin Fe per litre represents 2·2 cu. mm. of oxygen per c.c. when fully saturated, so 5·0 c.c. of a solution $H \times 10^{-4}$ g. atoms haemoglobin Fe per litre (the concentration used in the experiment) represents $11 \times H$ cu. mm. oxygen when fully saturated. The time t in seconds which would be required for complete saturation can be measured from the curve. The dry weight of 0·4 c.c. chloroplast suspension taken is 8·6 mg. The Q_{O_2} is thus $11 \times H \times \frac{3600}{t} \times \frac{1}{8\cdot6}$ cu. mm./mg. dry weight/hr.

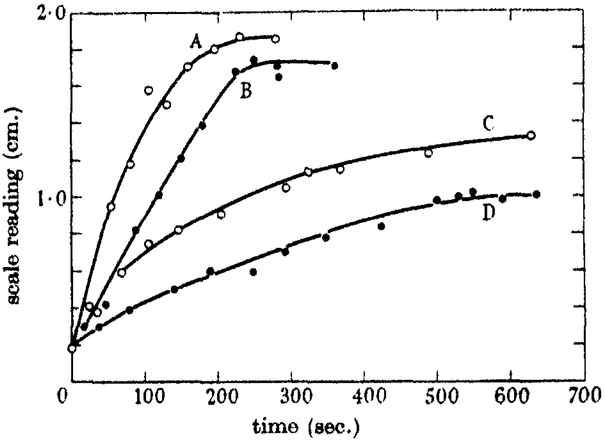

FIGURE 1. Experimental curves showing oxygen evolution by chloroplasts in ferric oxalate exposed to different light intensities. Haemoglobin $0\cdot7 \times 10^{-4}$, Fe 4×10^{-4}, chloroplast suspension 0·4 c.c. Curve *A*, light intensity, 40,300 lux; curve *B*, 18,600 lux; curve *C*, 6200 lux; curve *D*, 2070 lux.

4. MEASUREMENT OF REDUCTION OF IRON BY CHLOROPLASTS

The reagent for ferrous iron, $\alpha\alpha'$-dipyridyl (Hill 1930), was not suitable because the iron is reduced in presence of this substance by a variety of organic compounds.

Methaemoglobin, however, is reduced in the presence of oxygen by ferrous potassium oxalate giving haemoglobin which oxygenates immediately and the oxyhaemoglobin can easily be measured. As the experiments can be performed in the presence of atmospheric oxygen they can be carried out more rapidly and with less mechanical injury to the chloroplasts. In this method any oxygen production by the chloroplasts is neglected and the activity measured in terms of rate of reduction of methaemoglobin.

The methaemoglobin was prepared as follows. The stock solution of oxyhaemoglobin containing 1·7 g. atoms of Fe per litre was brought to pH 6·5 and treated with 1·2 vol. of 97 % alcohol. The bright red precipitate of parahaematin was filtered and thoroughly washed with distilled water until free from alcohol. The washed precipitate was suspended in a small volume of water and N/1 NaOH added until a viscous red solution was obtained (pH 11–11·5). After standing for 20 min. at room temperature the solution of alkaline methaemoglobin was brought to pH 6·5 with N/10 HCl. The denatured protein was thus precipitated, and the strong solution of methaemoglobin, completely free from oxyhaemoglobin, could be preserved unchanged for several weeks. The iron solution and potassium oxalate are the same as for the oxyhaemoglobin method. The mixture is made in the same manner using methaemoglobin instead of haemoglobin.

The mixture was placed in an open tube 1·5 cm. in diameter and illuminated after the addition of 0·4 c.c. of a chloroplast suspension. Methaemoglobin and oxyhaemoglobin of known concentration were placed in the cups of the colorimeter. The experiment is followed by reading the concentration of oxyhaemoglobin formed. The Q_{O_2} value is arrived at in the same way as for the oxygen method, only is here divided by a factor of 4. A typical experiment is shown in figure 2 giving the rate of oxyhaemoglobin production for four different light intensities; it will be noticed that the points lie on straight lines. Methaemoglobin requires 1 equivalent of hydrogen for its reduction to haemoglobin, while the oxygen combined as oxyhaemoglobin is equal to 4 equivalents of hydrogen per Fe. This is represented as follows:

$$4\mathrm{Fe}^{\cdot\cdot\cdot} \rightarrow 4\mathrm{Fe}^{\cdot\cdot} + \mathrm{O}_2,$$
$$4\mathrm{Fe}^{\cdot\cdot} + 4\mathrm{MetHb} \rightarrow 4\mathrm{Fe}^{\cdot\cdot\cdot} + 4\mathrm{Hb},$$
$$4\mathrm{Hb} + 4\mathrm{O}_2 \rightarrow 4\mathrm{HbO}_2.$$

Thus $4HbO_2$ measured represents 4 atoms of Fe reduced and is equivalent to 1 O_2 formed by the chloroplasts, and the HbO_2 is derived from the atmospheric oxygen in the fluid. In all tables, etc., the unit of concentration is gram atoms of iron per litre.

Blood methaemoglobin, more easily prepared, is less reactive towards ferrous potassium oxalate than muscle methaemoglobin. With concentrations of Hb $0{\cdot}8 \times 10^{-4}$ and $Fe^{\cdots}$ 4×10^{-4} and chloroplasts 0·4 c.c., the Q_O with blood MetHb was 3·6, with muscle MetHb 7·9.

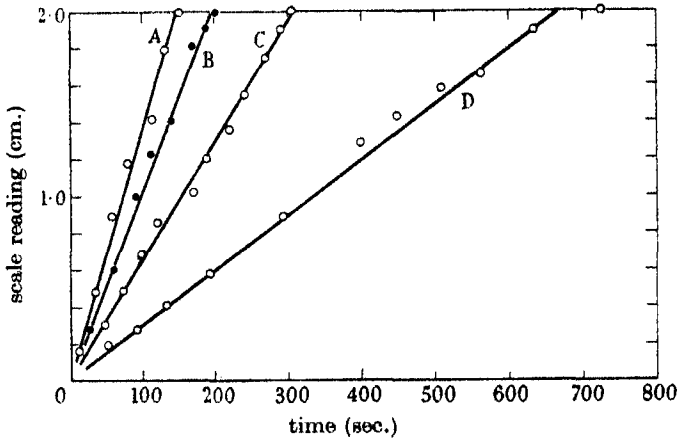

Figure 2. Experimental curves showing reduction of methaemoglobin by chloroplasts in ferric oxalate exposed to different light intensities. Methaemoglobin 1×10^{-4}, Fe 4×10^{-4}, chloroplast suspension 0·4 c.c. Curve *A*, light intensity, 40,300 lux; curve *B*, 18,600 lux; curve *C*, 6200 lux; curve *D*, 2070 lux.

5. Conditions affecting chloroplasts

(*a*) *Storage of suspensions*

The chloroplasts of *S. media* after isolation in sucrose phosphate buffer will keep their activity as measured by the methaemoglobin method for some time. Their ability to reduce ferric oxalate in light is, however, very labile; it is lost by mechanical agitation, e.g. after 6 min. shaking no activity or only very feeble activity could be detected. Boiling results in a complete loss of activity. On the other hand, they can be kept in a tube from which most of the oxygen has been removed without losing more activity than they would in air. No change in appearance could be seen when complete loss of activity had occurred. The loss of activity of chloroplasts on keeping is shown in figure 3. The linear nature of the initial loss of activity with time allows a correction to be made when a series of experiments is performed with the same sample of chloroplasts over a period of 1½ hr. It was noticed that chloroplasts of *S. media* plants from different localities showed different rates of loss of activity. Seeds collected from plants growing in these localities were sown and the plants grown under similar conditions in a greenhouse. The differences of rates of loss of activity were still found and are shown in figure 3. The strains are designated Q, K, H, N, and it is seen from the figure that the strain Q keeps its activity best; this strain was used for the experiments.

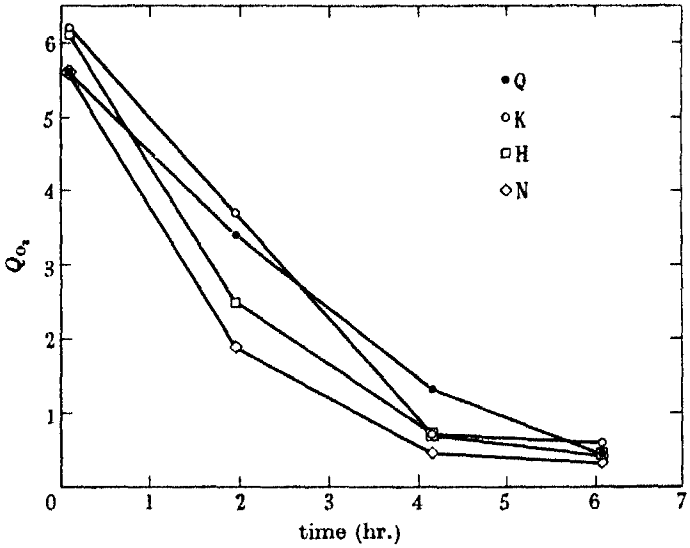

FIGURE 3. Differences in strains of chloroplasts, effect on activity of keeping the suspensions. Methaemoglobin concentration 1×10^{-4}, Fe 4×10^{-4}, chloroplast suspension 0·4 c.c.

(*b*) *Chloroplast concentration*

Figure 4 shows the effect of varying chloroplast concentration at three concentrations of methaemoglobin. The values for the Q_{O_2} are plotted against the $\log_{10}$ of the chloroplast concentration expressed as the molarity of the chlorophyll $(a+b)$ present. The Q_{O_2} is seen to rise with diminishing chloroplast concentration, but in extremely dilute suspensions the values, as would be expected, fall off in an irregular manner. These results show that even with very little chlorophyll present (the fluid being only faintly coloured green) the Q_{O_2} still rises with dilution of the pigment, so that a condition of light saturation has not been reached in our present investigation.

(c) *Methaemoglobin concentration*

The effect of varying the concentration of methaemoglobin is shown in figure 5. For high concentrations of methaemoglobin a cell filled with an optically equivalent strength is inserted in the light passing through the

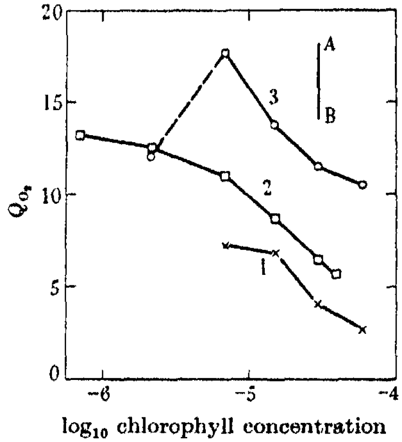

FIGURE 4. Effect of varying chloroplast concentration on Q_{O_2} at three concentrations of methaemoglobin. Fe 4×10^{-4}. Curve 1, methaemoglobin concentration $0{\cdot}8 \times 10^{-4}$. Curve 2, methaemoglobin concentration $1{\cdot}6 \times 10^{-4}$. Curve 3, methaemoglobin concentration $2{\cdot}4 \times 10^{-4}$. The chloroplast concentration is expressed as $\log_{10}$ chlorophyll concentration. The line *AB* denotes concentration normally used, i.e. 0·4 c.c. chloroplast suspension in 5 c.c. fluid.

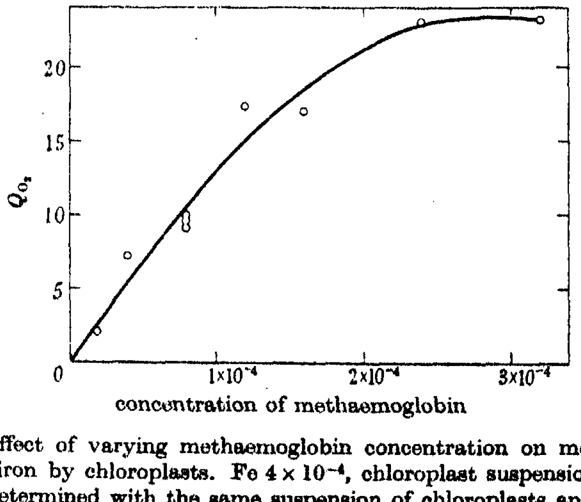

FIGURE 5. Effect of varying methaemoglobin concentration on measurement of reduction of iron by chloroplasts. Fe 4×10^{-4}, chloroplast suspension 0·1 c.c. All points were determined with the same suspension of chloroplasts and a correction made for loss of activity on keeping.

colorimeter cups, to correct for the excess of methaemoglobin over the oxyhaemoglobin contained in the lower cup. The Q_{O_2} rises with increasing concentration of methaemoglobin until the light intensity in the solution is reduced by the absorption of the methaemoglobin. At this point the blank experiment (without chloroplasts) is reduced nearly to zero, while the Q_{O_2} of the chloroplasts does not rise any higher. The rise of Q_{O_2} observed on increasing the concentration of methaemoglobin is due to the fact that the reaction between ferrous iron and methaemoglobin is a slow reaction. The velocity of the reaction

$$\text{Fe}^{\cdot\cdot} + \text{MetHb} \rightarrow \text{Fe}^{\cdot\cdot\cdot} + \text{Hb}$$

can be measured when oxygen is present by adding a ferrous salt to a buffered solution (pH 7·9) of methaemoglobin containing oxalate and directly observing with a stopwatch the rate of change in intensity of the oxyhaemoglobin spectrum, as in the case of the experiments with chloroplasts. By the mixing of 4×10^{-4} $\text{Fe}^{\cdot\cdot}$ with a solution of methaemoglobin 1×10^{-4} a velocity constant for the appearance of oxyhaemoglobin of 0·6 mol.$^{-1}$/sec. is obtained. This is a very slow reaction compared with the combination of muscle haemoglobin with oxygen which was found by Millikan (1936) to be 19,000 mol.$^{-1}$/sec. If the experiments on the reduction of methaemoglobin by ferric iron and chloroplasts are performed in intermittent light the Q_{O_2} observed is thereby raised. The following experiment (table 1) with a low rate of flashing illustrates the effect.

TABLE 1. INTERMITTENT LIGHT AND THE REDUCTION OF METHAEMOGLOBIN BY CHLOROPLASTS

MetHb 1×10^{-4}, $\text{Fe}^{\cdot\cdot\cdot}$ 6×10^{-4}, light period 1/9 × dark period. 21 flashes per sec.

	Q_{O_2}
Continuous light 18,600 lux	4·1
Light decreased to 1/9 by screen	0·9
Light decreased to 1/10 by rotating sector	2·7

(*d*) *Ferric oxalate concentration*

The effect of increase in iron concentration on the ferric oxalate reaction as measured by the evolution of oxygen shows no regular increase of Q_{O_2}. The results of experiments at higher concentrations of iron than 6×10^{-4} were inconsistent.

When the reduction of methaemoglobin is measured the rate of reduction increases regularly with $\text{Fe}^{\cdot\cdot\cdot}$ at low concentrations. With high iron concentrations the activity of the chloroplasts appears greatly reduced, and at the same time there is a large blank—that is, the methaemoglobin is reduced in light without chloroplasts being present. Ferric oxalate itself is broken up by exposure to light forming ferrous iron at the expense of organic material present. At the usual low concentrations of iron used in the experiments this effect of light on ferric oxalate itself is negligible compared with the rate of reduction of iron catalysed by the chloroplasts. The illumination of strong ferric potassium oxalate, however, has been found to have a destructive effect on haemoglobin when it is present as shown by the formation of a precipitate of denatured protein. This destructive effect on protein would explain the falling off in activity of the chloroplasts in high concentrations of ferric oxalate. Table 2 shows the results, and it is clear that when the blank experiment without chloroplasts begins to increase the activity of the chloroplasts is reduced.

TABLE 2. IRON CONCENTRATION AND REDUCTION OF METHAEMOGLOBIN

MetHb $1{\cdot}2 \times 10^{-4}$, chloroplasts 0·4 c.c. The blank is given as a Q_{O_2} on the basis of the chloroplasts used in the experiment.

$Fe^{\cdots}$ conc.	Q_{O_2}	'Q_{O_2}' no chloroplasts
0	0·20	—
$0{\cdot}4 \times 10^{-5}$	0·88	—
$1{\cdot}2 \times 10^{-4}$	1·4	—
$2{\cdot}4 \times 10^{-4}$	3·7	—
$4{\cdot}0 \times 10^{-4}$	2·8	—
$4{\cdot}0 \times 10^{-4}$	2·6	—
$4{\cdot}0 \times 10^{-4}$	2·8	—
$4{\cdot}0 \times 10^{-4}$	3·3	—
$20{\cdot}0 \times 10^{-4}$	6·6	0·3
$150{\cdot}0 \times 10^{-4}$	5·6	1·6
$400{\cdot}0 \times 10^{-4}$	4·6	2·6

In order to make comparison easy, the reduction of methaemoglobin without chloroplasts in the controls has been expressed as a Q_{O_2} calculated on the basis of the chloroplasts used in the experiments.

(e) *Temperature*

Rise in temperature increases the activity of the chloroplasts measured by both the haemoglobin and methaemoglobin methods, as shown in tables 3 and 4 respectively. The effect of temperature appears greater for the reaction involving the reduction of methaemoglobin. Direct experiments showed that on adding ferrous potassium oxalate to methaemoglobin the rate of reaction increases with rise of temperature, and this would therefore account for the higher Q_{10} observed for the ferric oxalate reaction measured by methaemoglobin. These results, however, are both concerned with the effect of temperature change on the reagents used rather than on the activity of the chloroplasts.

TABLE 3. TEMPERATURE AND EVOLUTION OF OXYGEN

Hb 1×10^{-4}, Fe''' 6×10^{-4}, chloroplasts 0·4 c.c.

Temp. °C	Q_{O_2}	Q_{10}
19	15·6	1·3
0	9·5	
19	24·0	1·4
0	11·6	
23	16·2	1·3
0	9·4	
Hb $0{\cdot}8 \times 10^{-4}$, Fe''' 8×10^{-4}, chloroplasts 0·4 c.c.		
30	11·0	1·4
0	4·4	

TABLE 4. TEMPERATURE AND REDUCTION OF METHAEMOGLOBIN

MetHb 1×10^{-4}, Fe''' 4×10^{-4}, chloroplasts 0·4 c.c.

Temp. °C	Q_{O_2}	Q_{10}
20	5·1	1·7
30	8·7	
20	5·1	1·5
30	7·5	
20	4·0	1·9
10	2·1	
10	2·1	1·8
0	1·2	

(*f*) *Hydrogen-ion concentration*

In this case, as in the last section, the reagents used complicate the effect of varying pH. The maximum activity seems to be reached at pH 8; more alkaline than this the activity of the chloroplasts is destroyed. On the acid side the activity measured falls to half its value at pH 6·5 and to zero in the region pH 5·6.

(g) *Light intensity*

The effect of varying light intensity on the ferric oxalate reaction as measured by oxygen production is shown in table 5. The results are summarized in the graph (figure 6). Owing to the ease with which the chloroplasts are injured by mechanical means, the upper limit of values may be nearer the actual form of the light curve than the mean values. In the case of the reaction measured by the reduction of methaemoglobin there is less agitation of the chloroplast suspension and the results show less variation, although the actual Q_{O_2} values are lower; this is shown in figure 7 and table 6.

TABLE 5. LIGHT INTENSITY AND EVOLUTION OF OXYGEN

Hb $1{\cdot}0 \times 10^{-4}$, $Fe^{\cdot\cdot\cdot}$ 4×10^{-4}, chloroplasts 0·4 c.c. The figures refer to Q_{O_2}.

Light intensity			
40,300 lux	18,600 lux	6200 lux	2100 lux
8·2	10·9	14·0	5·4
19·2	13·6	9·6	2·9
25·1	15·2	3·9	3·1
—	13·0	7·6	2·8
—	10·0	8·6	4·6
—	15·6	6·0	2·1
—	17·6	12·9	3·9
—	—	—	4·4
Mean 17·5	13·7	8·9	3·6

TABLE 6. LIGHT INTENSITY AND REDUCTION OF METHAEMOGLOBIN

MetHb $0{\cdot}7 \times 10^{-4}$, $Fe^{\cdot\cdot\cdot}$ 4×10^{-4}, chloroplasts 0·4 c.c. The figures refer to Q_{O_2}.

Light intensity			
40,300 lux	18,600 lux	6200 lux	2100 lux
—	4·7	2·6	0·8
5·0	3·8	2·6	0·9
5·0	4·1	2·8	1·2
5·3	3·8	2·6	1·1
Mean 5·1	4·1	2·6	1·0

The form of the curves obtained when Q_{O_2} is plotted against light intensity shows similarity to those obtained by various workers on the assimilation of the living plant (Smith 1936). The position of the curve in relation to light intensity lies in the range of values found with different plant materials. There is one point of difference, that in both methods, i.e. oxygen production and the reduction of methaemoglobin, the curve is never parallel to the axis even at the high light intensity used. In the experiments with progressive dilution of chloroplasts at a high light intensity (figure 5) it was found that the Q_{O_2} continued to rise, showing that under our conditions the state of 'light saturation' was not reached.

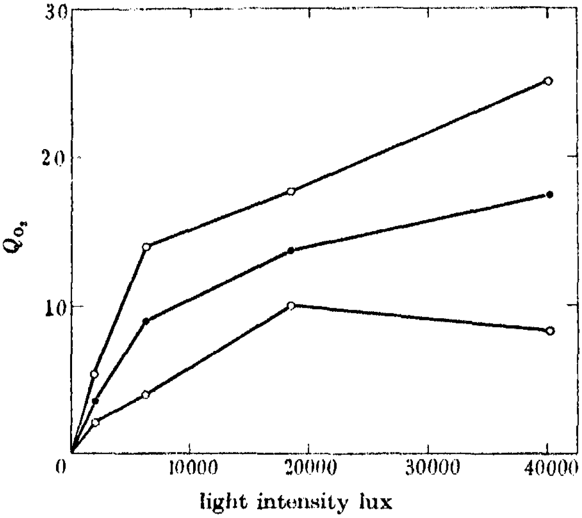

FIGURE 6. Effect of varying light intensity on rate of evolution of oxygen by chloroplasts. Fe 4×10^{-4}, chloroplast suspension 0·4 c.c. Circles, extreme values; dots, mean value.

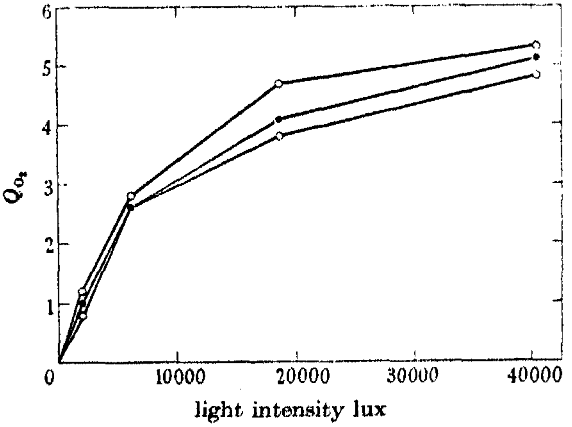

FIGURE 7. Effect of varying light intensity on the rate of reduction of methaemoglobin by chloroplasts. Fe 4×10^{-4}, methaemoglobin $0·7 \times 10^{-4}$, chloroplast suspension 0·4 c.c. Circles, extreme values; dots, mean values.

(*h*) *Inhibition by urethane*

Whereas sodium azide, fluoride, cyanide, and hydroxylamine have no effect on the evolution of oxygen from ferric oxalate by chloroplasts (Hill 1939), urethane has a marked inhibitory effect. The inhibition increases with time of keeping the chloroplasts in urethane. With 3 % ethyl urethane the inhibition is reversible, but with 10 % ethyl urethane the chloroplasts appear swollen, lose their silky appearance when in suspension, and do not return to normal after removal from urethane. The inhibitions produced by ethyl and phenyl urethanes are shown in tables 7–10. A standard time of incubation with urethane of 6 min. was chosen.

TABLE 7. ETHYL URETHANE AND EVOLUTION OF OXYGEN

Hb $0{\cdot}7 \times 10^{-4}$, $Fe^{\cdots}$ 4×10^{-4}, chloroplasts 0·4 c.c. Fresh suspension prepared for each pair of experiments.

Ethyl urethane conc. %	Q_{O_2}	Inhibition %
0	14·8	80
2	3·0	
0	12·7	60
1	5·1	
0	14·4	43
0·5	8·2	
0	20·0	26
0·2	14·8	

TABLE 8. PHENYL URETHANE AND EVOLUTION OF OXYGEN

Hb $0{\cdot}7 \times 10^{-4}$, $Fe^{\cdots}$ 4×10^{-4}, chloroplasts 0·4 c.c. Fresh suspension prepared for each pair of experiments.

Phenyl urethane conc. %	Q_{O_2}	Inhibition %
0	16·4	90
0·02	1·6	
0	6·2	84
0·004	1·0	
0	23·0	90
0·02	2·4	
0	20·0	80
0·004	3·9	
0	18·0	63
0·0008	6·6	
0	15·1	4
0·0002	14·5	

Phenyl urethane inhibits the chloroplast reaction in much lower concentrations than ethyl urethane. This is in agreement with the inhibition of photosynthesis found by Warburg (1919, 1920) for a homologous series of urethanes (table 11).

TABLE 9. ETHYL URETHANE AND REDUCTION OF METHAEMOGLOBIN

MetHb 1×10^{-4}, $\mathrm{Fe}^{\cdots}$ 4×10^{-4}, chloroplasts 0·4 c.c. Suspensions were kept for several experiments and corrected for loss of activity on standing. The correction is based on loss of activity at the rate of 0·6 Q_{O_2} per hour.

Ethyl urethane conc. %	Q_{O_2}	Time min.	Q_{O_2} uninhibited	Inhibition %
0	4·0	0	4·0	—
1·0	3·7	11	3·9	5
2·0	2·3	18	3·8	40
3·0	0·3	24	3·7	92
0	3·5	37	3·6	—
0	4·4	0	4·4	—
1·5	2·3	30	4·1	44
2·5	1·4	37	4·0	65
0	3·9	50	3·9	—

TABLE 10. PHENYL URETHANE AND REDUCTION OF METHAEMOGLOBIN

MetHb 1×10^{-4}, $\mathrm{Fe}^{\cdots}$ $4{\cdot}0 \times 10^{-4}$, chloroplasts 0·4 c.c. Suspension kept for several experiments and corrected for loss of activity on standing. The correction is based on loss of activity at the rate of 0·6 Q_{O_2} per hour.

Phenyl urethane conc. %	Q_{O_2}	Time min.	Q_{O_2} uninhibited	Inhibition %
0	3·8	0	3·8	—
0·005	2·1	5	3·75	44
0·015	0·8	54	3·3	76
0·025	1·9	23	3·6	47
0	3·5	33	3·5	—
0·040	0·67	10	3·7	82
0·060	0·33	44	3·4	90
0	3·2	64	3·2	—

TABLE 11. COMPARISON OF MOLECULAR INHIBITION BY URETHANE

Concentration of urethane required for 50 % inhibition of activity
(millimols per litre).

	Ethyl urethane	Phenyl urethane
Photosynthesis (Warburg)	220	0·5
Ferric oxalate reaction:		
Oxygen evolution	60	0·04
Reduction of methaemoglobin	180	0·5

6. Discussion

The activity of isolated chloroplasts of *Stellaria media* has been measured by two methods. The first method involves the direct measurement as oxyhaemoglobin of molecular oxygen produced by the system. The second method is the indirect measurement of the iron reduced through its reaction with methaemoglobin. The second method is preferable from an experimental point of view, but it gives lower values for the activity of the chloroplasts than the measurement of oxygen. The low values of activity were shown to be due to the slowness of the reaction of ferrous oxalate and methaemoglobin, so that it is this reaction which limits the rate observed. This is seen in the measurements of urethane inhibition; the activity measured by evolution of O_2 appears more sensitive to the inhibitor than when measured by the reduction of methaemoglobin by ferrous oxalate. The inhibitor then must act on the mechanism producing molecular oxygen and causing the reduction of ferric oxalate.

It has been thus shown that the ferric oxalate reaction of chloroplasts has certain of the characteristics of the photochemical reaction in carbon assimilation. Further the photochemical activity of the isolated chloroplasts of *S. media* has been shown to be relatively high. Calculated on the basis of the dry weight of leaves, the maximum positive Q_{O_2} obtained is above 20. In this reaction with ferric potassium oxalate the molecular oxygen produced is measured directly by means of haemoglobin. Molecular oxygen is one of the products of normal photosynthesis and also is not a common product of photochemical reactions.

It is therefore assumed that the measured activity of the system in the isolated chloroplast responsible for the production of oxygen in light represents a part of the process of normal photosynthesis.

The ferric oxalate reaction with chloroplasts resembles photosynthesis in producing molecular oxygen but differs essentially in that carbon dioxide does not act as a hydrogen acceptor. The inhibition by urethane is closely similar for photosynthesis in chlorella cells and isolated chloroplasts of *Stellaria*; cyanide and hydroxylamine, while they inhibit photosynthesis, do not prevent the production of oxygen with ferric oxalate. If, then, the ferric oxalate reaction corresponds with the photochemical reaction these facts are in agreement with the conclusions of Warburg from his work on *Chlorella*: urethane affected the photochemical reaction while cyanide affected the dark reaction in which carbon dioxide was involved. The new conclusion that can be drawn from the work on isolated chloroplasts is that oxygen itself is formed in a photochemical reaction during which there is no reaction involving carbon dioxide.

We are indebted to the Government Grants Committee of the Royal Society for the greenhouse used in this work. We are very grateful to the Research Department of the British Thomson Houston Company for supplying data of the illumination produced by one of their projection lamps. One of us (R. S.) received a maintenance grant from the Department of Scientific and Industrial Research for part of this work, and for the remainder a grant from the Salters' Company.

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The mechanism of the semicircular canal. A study of the responses of single-fibre preparations to angular accelerations and to rotation at constant speed

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Single-fibre discharges have been recorded from the horizontal ampulla of the isolated labyrinth of *Raja*. A basis is provided for the interpretation of labyrinthine tone, and of the reflex responses to rotation of unilaterally operated animals. The sensory activity of the semicircular canal provides a framework accounting for nystagmus and after-nystagmus.

Introduction

When an annular tube filled with fluid is rotated in its own plane, the fluid, owing to its inertia, stays behind for a brief interval of time at the beginning of rotation, and thus there results a momentary flow of fluid relative to the wall of the tube in a direction opposite to that of rotation. This principle was adduced by Mach (1875) to explain the mode of action of the semicircular canals, and, in the amplified form given to it by Breuer (1891), who discussed the deformation of the sensory hair cells in the ampulla resulting from the flow of endolymph, the theory has generally served as the basis of subsequent consideration of the function of the semicircular canals. Analysis of labyrinthine reflexes has, however, disclosed a complexity of function which is not easily reconciled with so simple a scheme, and in the recent literature, although the Mach-Breuer theory has not been set aside, at least so far as concerns the mechanism of the semicircular canals, its precise application to the interpretation of a growing body of experimental knowledge has proved difficult and uncertain. This knowledge is derived to a great extent from experimental eliminations of either or both labyrinths, or of separate parts of the labyrinth.

Removal of both labyrinths produces a loss of tone in the neck and limb musculature and in the eye muscles, and when the operation is unilateral the resulting tonic asymmetry is the more striking. This phenomenon was first observed by Ewald (1892) who described the semicircular canals as the seat of the tonic function of the labyrinth. Later, especially under the influence of the work of Magnus and de Kleijn, the otolith apparatus came to be regarded as the main source of labyrinthine tone. However, a number of workers (Maxwell 1923; Lorente de Nó 1926; Huizinga 1932, 1935; Tait and McNally 1934; Löwenstein 1937) have furnished conclusive evidence that the semicircular canals have an important share in the influence which the labyrinths exercise over muscle tone. What is the nature of this influence? Elimination experiments alone can yield no more precise definition than is implied in Huizinga's supposition of 'eine gewisse Spannung in den Cristae'. Only oscillographic analysis of the sensory activity of the canals can hope to advance our understanding of the tonic influence a stage further.

The compensatory responses of limbs and eyes to rotatory displacement of the animal, being antagonistic with respect to opposite senses of rotation, have naturally pointed to an antagonistic action of opposite pairs of semicircular canals. We have discussed the integrated action of the canals elsewhere (Löwenstein and Sand 1940). Here we will recall merely that evidence has accumulated to show that a single intact horizontal canal can be excited by both senses of horizontal rotation to evoke the appropriate nystagmus. Indeed, Lorente de Nó (1931) insists that ampullopetal and ampullofugal flow of endolymph produce reflex effects of equal magnitude. His views are based on experiments with mammalian eye muscles. On the other hand, McNally and Tait (1933), studying the limb responses of frogs, adhere to Crum Brown's (1874) contention that each member of a functionally associated pair of canals responds to rotation in one sense only. This view was also supported by Ross (1936), who was able to obtain oscillographic records of action potentials occurring in slender filaments of the eighth cranial nerve of the frog during rotary stimulation of the isolated labyrinth. Ross stated, however, that the technique employed in these experiments did not allow to rule out altogether the possibility of a two-way response of the cristae. A further difficulty arises in consideration of the time relations of nystagmus and after-nystagmus. Maier and Lion (1921) found that on an electrically driven turntable nystagmus ceased (in man) after 20 to 40 sec. of constant rotation, and that after prolonged rotation after-nystagmus lasted 20–40 sec., and Masuda (1922) showed that, in the guinea-pig, the duration of after-nystagmus is proportional to the angular velocity of rotation, increases with the duration of rotation and attains a maximum after 20–40 sec. of rotation. Why always 20–40 sec.? it may be asked. At first sight the problem of reconciling these facts with any peripheral mechanism based on the Mach-Breuer concept appears hopeless, for it must be recalled that displacement of the endolymph can occur only during angular *acceleration*, not during rotation at constant speed, and that, therefore, once the initial acceleration from rest has taken place, the continuation of a constant rotation can exert, apparently, no further influence upon the canal and its contents. It was on the ground of these considerations that Mach maintained that the peripheral excitation during a rotation can be only momentary, and Bárány (1907) argued that the duration and frequency of nystagmus are determined by prolonged central excitatory processes evoked by a brief sensory excitation in the crista. More recently, Lorente de Nó (1931) appears to have accepted this view, and Mowrer (1935) has brought forward experimental evidence in support of it.

In short, there are three aspects of the problem of the mechanism of the semicircular canals which require elucidation: (1) the nature of the tonic influence, (2) the mechanism of discrimination of antagonistic senses of rotation by an individual crista, (3) the causes of the time relations of nystagmus and after-nystagmus.

New light has been shed on the mechanism of the semicircular canal by Steinhausen (1931, 1933, 1935), who, by means of an elegant technique, disclosed to view the cupula in the ampullae of surviving preparations of the labyrinth of the pike, and recorded photographically its movements during rotation as well as the accompanying reflex responses of the eyes. It is true that the existence of the cupula as a living structure has been denied by some histologists, and in recent times Bowen (1933) has again insisted that the cupula is in reality a coagulation product. But in the same communication he writes, 'with dark-field illumination there was frequently found above the crista a material which appeared slightly more mucilaginous than the fluids of the neighbouring endolymphatic spaces'. No more distinctive features are required to establish the cupula as a real and, indeed, fundamentally important structure in the sensory apparatus of the semicircular canal. As Steinhausen's observations have revealed, the cupula is a jelly mass capping the crista, filling the whole cross-section of the ampulla, and containing within itself the sensory hair processes of the crista. When hydrostatic pressure is applied to the canal, the endolymph is free to flow along it. Not so the cupula, which, at its proximal end, adheres to the crista, and is not free to depart from it. Its distal end, however, is free to circumscribe the curved wall of the ampulla. Thus the action of the cupula is like that of a swing-door, it sweeps from one side of the ampulla to the other in obedience to the forces operating upon it through the endolymph. Steinhausen's photographic recordings, which were shown at the 16th International Congress of Physiology at Zürich in 1938, have demonstrated this with great clarity. They have demonstrated further that the cupula is not a formless mass, but possesses elasticity by virtue of which it returns again when deflected from its normal position of rest. The cupula is in fact a highly damped jelly pendulum and its period is in the neighbourhood of 20 sec. (Steinhausen 1933).

These facts have a profound significance in the definition of the sensory properties of a semicircular canal. The cupula and the sense hairs are mechanically a single unit, and the mechanical properties governing the displacement of the cupula must determine also the deformation of the sense hairs from which arises the excitation of the end organ. The manner of this excitation and the form of the afferent discharges that result from it have always been the ultimate consideration in all discussion of labyrinth activity. Until recently these discharges have not been accessible to direct experimental investigation. Four years ago we made our first attempt to obtain oscillographic records from the nerve-branch to the horizontal ampulla of the dogfish (Löwenstein and Sand 1936). We obtained evidence that the ampulla discharges impulses spontaneously when at rest, and that this discharge is augmented during ipsilateral rotation and diminished during contralateral rotation. But our records gave only an additive picture of asynchronous activity in the whole nerve branch, and, since at that time it seemed impossible to record the activity of single units, our results were necessarily no more than qualitative. Ross (1936) obtained single fibre discharges from the eighth nerve of the frog, but did not analyse them quantitatively. His interpretation of the mode of function of the semicircular canals differs fundamentally from that offered by us, as he does not envisage a spontaneous impulse discharge as a background for a two-way response of the ampullary sense ending. Ross did observe impulses 'appearing in the filament for no good or obvious reason' and 'a resting level of random activity', but he remained doubtful as to 'whether or not this type of activity is characteristic of the normal labyrinth'. Indirect but none the less significant confirmation was provided by a study of lateral-line receptors (Sand 1937). Here single-fibre discharges were successfully recorded and the same picture of a spontaneous rhythm of discharge which could be accelerated and inhibited by antagonistic stimulation emerged with greater clarity. Another group of elasmobranch sense organs, the ampullae of Lorenzini, were shown to have an analogous mode of behaviour (Sand 1938). Our success in recording the activity of individual semicircular canals in surviving preparations of the isolated elasmobranch labyrinth (Löwenstein and Sand 1940) has encouraged us to attempt the quantitative analysis of single-fibre discharges from the horizontal ampulla in response to known angular accelerations and rotations at constant speed. This information is crucial for testing any hypothesis of the mechanism of canal activity, and although the difficulty of teasing out single active units in a nerve twig not more than 2 mm. long, attached at one end to the delicate crista, and lying on the thin wall of the utriculus, is considerable, we have had a sufficient number of successful preparations to furnish the information required. This information, as will presently appear, confirms Steinhausen's observations on the cupula in a most striking manner, and provides a basis for the interpretation of labyrinthine tone, of the two-way response of an individual canal, and of the time relations of nystagmus and after-nystagmus.

METHODS

The isolated labyrinth of *Raja clavata* is prepared by pithing a ray of medium size (about 20 in. wing span), removing the lower jaw, severing the head and dividing it into left and right halves by an accurate section in the median vertical plane. The eye is then excised, the rostrum and muscles removed, and the preparation cleaned so as to leave only the cartilaginous labyrinth. The ampulla of the horizontal canal and its nerve branch can now be located under a binocular through the posterior wall of the orbit, and by shaving away a groove of cartilage the nerve and lower margin of the ampulla are exposed. The electrode holder consists of two rectangular blocks of ebonite assembled in the form of an L. On its base is mounted a micromanipulator carrying the grid electrode. The straight edges of the roof and floor of the cranium left by the median section through the head can be applied to the vertical surface of the electrode holder, and thus the labyrinth, when secured by rubber bands, can be mounted accurately in its normal horizontal position. Under the binocular, and with very fine instruments, the nerve is divided into strands, care being taken not to perforate the delicate utricular wall upon which the nerve lies. Subdivision of the nerve combined with gentle partial crushing close up to the ampulla is pushed as far as possible in the hope of isolating a single unit. A watchmaker's forceps, mounted on the micromanipulator, serves as grid electrode, and when the dissection is complete the peripheral stump of the nerve strand is gripped in the forceps and lifted from the utricular wall. The neutral electrode, a silver wire, is laid on a wisp of cotton-wool soaked in Ringer against the cartilage close to the opening, and held there by the rubber bands securing the preparation. A few threads of cotton-wool make contact with the edge of the ampulla so as to reduce the inter-electrode resistance as nearly as possible to the value of the nerve resistance itself. The holder is placed on a small wooden turntable, 16 cm. diameter, with a long pivot running in oil, and carrying, under the turntable, a small pulley to which the drive is applied. The position of the holder on the turntable is so adjusted that the labyrinth is placed approximately at the centre of rotation. The input electrodes are connected to two terminals placed at the top edge of the holder, and from here a short length of flex connects with the input leads to the amplifier. As it was rarely necessary to rotate more than half a revolution no difficulty was experienced from the twisting of the flex.

Angular displacement of the turntable was recorded simultaneously with the action potentials by means of a slotted disk mounted concentrically on the turntable and interrupting a light beam shining on to a photocell. The disk was of brass, 30 cm. diameter, and was cut with three rows of slots. The three rows contained 120, 30 and 10 slots respectively round the circumference, thus providing intervals of 3, 12 and 36°. The photocell was connected to a single-stage amplifier operating a sensitive relay in series with a 2·5 V bulb which provided a light spot reflected into the camera. The relay was so connected that when the light beam to the photocell was interrupted by the disk the light spot shone into the camera, and when the beam passed to the photocell through a slot in the disk the light spot was extinguished. Frequency of signals is limited by the inertia of the relay, on the other hand it is desirable to have as many signals as possible on the record for each rotation. The slot interval suitable for a given acceleration had to be chosen with some care. To avoid interference from the signal it was important that its 2·5 V supply should be insulated from earth. Figure 1 shows a photograph of the turntable assembly.

Uniform angular *acceleration* was obtained by driving the turntable from a weight falling through a height of 6 ft. The arrangement used is illustrated in figure 2 and consists of two counterpoised weights suspended on fine cords running over pulleys. The drive is taken from the centre pulley through suitable reduction gearing. With one pan at the top and the other at the bottom the weight is placed on the top pan, and is held in position by a friction clutch applied to the fast pulley of the reduction gear. On releasing the clutch the weight falls and an acceleration is imparted to the turntable. The weight is prevented from striking the floor by applying the clutch again at the appropriate moment, and the turntable is brought quickly to rest. By transferring the weight from one pan to the other the direction of rotation of the turntable is reversed. As it was desired that the acceleration of the turntable should last about 3 sec. it was necessary to apply a suitable load to the centre pulley of the falling weight mechanism. This was achieved, very conveniently, by installing a $\frac{1}{8}$ h.p. high-speed motor with a heavy pulley, removing its brushes (so as to avoid a fluctuating frictional resistance) and causing it to be rotated passively by the revolution of the fast pulley of the reduction gear. The motor and the falling weight mechanism were thus connected in parallel, as it were, to the reduction gear. When it was desired to rotate the turntable at constant speed the drive from the falling weight mechanism was disconnected, the brushes re-inserted in the motor, which was then driven electrically in the usual way. The actual value of the falling weight had to be adjusted within rather narrow limits. It is limited at one extreme by the inertia of the whole assembly, and at the other by the frictional grip of the cord on the centre pulley of the falling weight mechanism. A weight of 520 g. was used.

FIGURE 1. *Photograph of turntable assembly. D*, slotted disk; *L*, isolated labyrinth; *P*, photocell; *S*, spot light.

All these provisions have to be made with considerable care, but when everything is properly adjusted it is surprising how well and uniformly the mechanism works. It is, of course, hardly to be expected that it should provide an angular *acceleration* of theoretical constancy, but the photoelectric signalling device enabled us to plot the movement of the turntable directly, and to obtain a satisfactory estimate of the value and constancy of the acceleration in every recording.

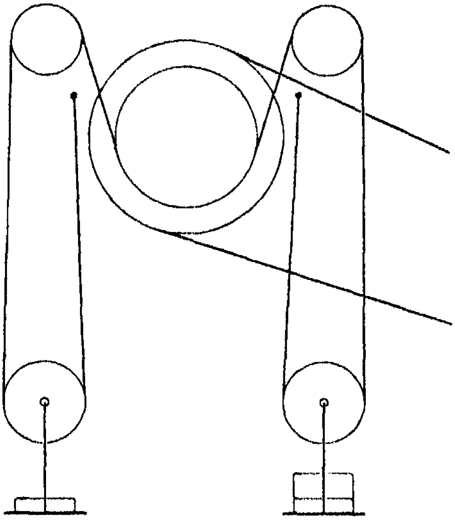

FIGURE 2. Diagram of falling weight mechanism.

Action potentials were recorded with a cathode-ray tube using a stationary spot whose deflexions were photographed on moving bromide paper. A high-gain, condenser coupled amplifier provided a maximum voltage amplification of 5,000,000. Small condensers were inserted in the balanced input stage, as we were concerned only with the frequency of spike potentials. The time signal was a synchronous motor driving a perforated disk in front of a light spot and giving 20 time marks a sec.

RESULTS

Several preparations were obtained in which the nerve had been sufficiently reduced to permit the identification of individual impulse trains with complete confidence. All of them confirmed our original finding of a spontaneous frequency of discharge which is increased and inhibited by ipsilateral and contralateral rotation respectively. Altogether more than a dozen recordings were made of responses to different speeds of acceleration in a clockwise and counterclockwise direction. A typical record is reproduced in figure 3. The large potentials are clearly derived from the activity of a single unit. The record shows the spontaneous frequency of discharge from the stationary labyrinth, the gradual increase in frequency throughout 3 sec. of ipsilateral acceleration, and the complete inhibition of the discharge during contralateral acceleration. The single impulse train appears against a background of smaller potentials, derived apparently from several fibres, whose frequency of discharge does not appear to be significantly influenced by angular acceleration. Such small potentials have been frequently noted, and their meaning remains obscure. One explanation would be that they are recorded from fibres less favourably situated with respect to the electrodes. If that were so it is to be expected that in some preparations these non-responding fibres would yield the dominant potentials. But this has not happened, except in one case where a pure single-fibre discharge was found to be quite unaffected by rotation. But in that case, it was found, on examination, that the horizontal canal was damaged and had collapsed—an observation which in itself provides an interesting confirmation of our general conclusions. In no instance have we obtained a record in which the non-responding potentials were dominant and the responding potentials in the background. This leads one to the alternative explanation that the ampulla, in addition to its principal innervation, is supplied with fibres of relatively small diameter which are not concerned with the response to rotation and whose function remains unknown. But whatever the significance of these small potentials may be the fact stands out with complete clarity that, whether one is recording from a nerve in which a large number of fibres are active together, or from one reduced to a point where the activity of an individual unit can be identified, the sensory activity of the organ is demonstrated by the increase and decrease in the frequency of a resting discharge.

The records of figure 3 were taken at a paper speed of 5·2 cm. per sec. for the purpose of reproduction. For quantitative analysis, however, we have recorded at a paper speed of 19 cm. per sec., and this gave records in which the angular displacement and frequency of impulses could be measured with great accuracy. From the measurements, the acceleration was calculated by applying the kinematic formula $a = 2\theta/t^2$, where θ is the angular displacement from rest in time t during a constant acceleration a. This process involves an error due to the uncertainty of the instant of the beginning of rotation. We took care to reduce this error to a minimum by adjusting the position of the turntable before each recording so that the light spot to the photocell impinged on the edge of the first slot through which it would fall, thus insuring that the first rotation signal on the record would be as near as possible to the actual beginning of the rotation.

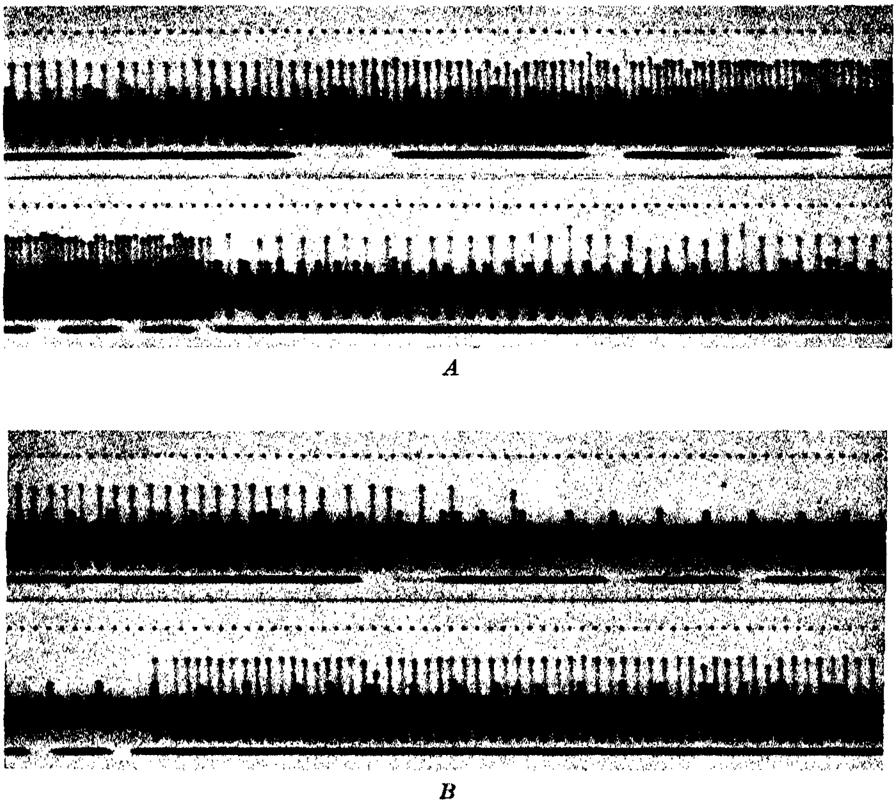

FIGURE 3. Oscillograph records from the nerve branch to the left horizontal ampulla, showing the response of a single unit to angular acceleration to the left (*A*) and to the right (*B*). The lower strip of each pair is the continuation of the upper. Time in 0·05 sec. is marked at the top of the record, and the acceleration is recorded by the signal line at the bottom. Successive gaps in the line mark the revolution of the turntable through 12°. The acceleration in record *A* is 15° per sec.^{2}; in record *B*, 20° per sec.^{2}.

Nevertheless, the error is considerable for the smaller values of θ and t, but becomes less as rotation proceeds. We have calculated the values of a from the greater values of θ in each record, and have convinced ourselves, by analysis of the data, that the acceleration did not vary by more than 20 per cent throughout a rotation.

In figures 4 and 5 are plotted the responses of the same preparation to ipsilateral rotation at an acceleration of 15° per $sec.^2$ and to contralateral rotation at an acceleration of 18° per $sec.^2$. It should be noted, in passing, that in figure 5, recorded a few minutes after figure 4, the resting discharge had returned to the same frequency as before the recording of figure 4,

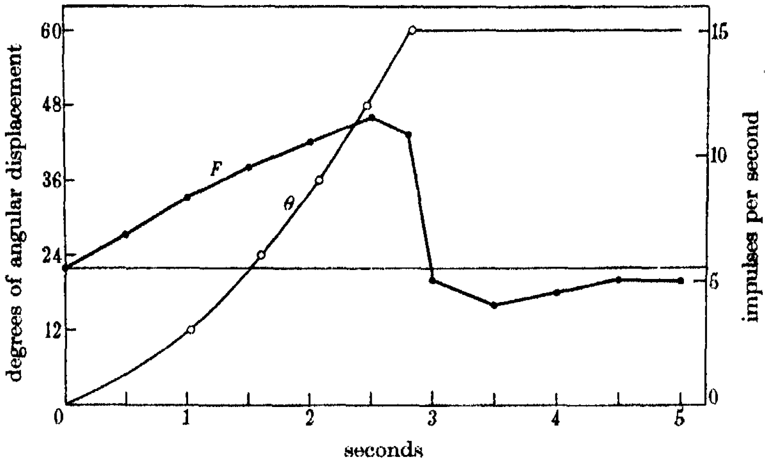

FIGURE 4. Frequency response of a single receptor unit of the left horizontal canal to ipsilateral (counterclockwise) turntable rotation at an acceleration of approximately 15° per $sec.^2$. *F*, frequency of discharge in impulses per sec. calculated as the reciprocal of the period between adjacent impulses. *θ*, degrees of angular displacement from rest. The horizontal line marks the frequency of the resting discharge.

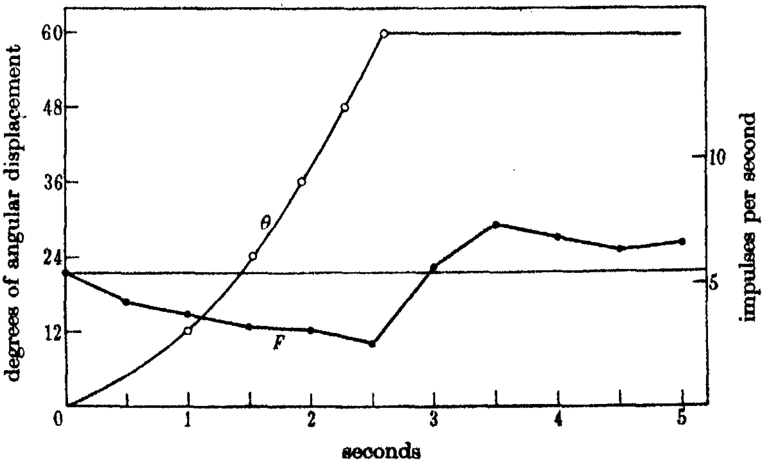

FIGURE 5. Same preparation as in figure 4. Response to contralateral (clockwise) acceleration at approximately 18° per $sec.^2$.

namely 5·5 sec. It should be noted also that the entire stimulation of the labyrinth to these determinations was no more than a turning through 60° in a little over 2·5 sec. The principal point that emerges from these results

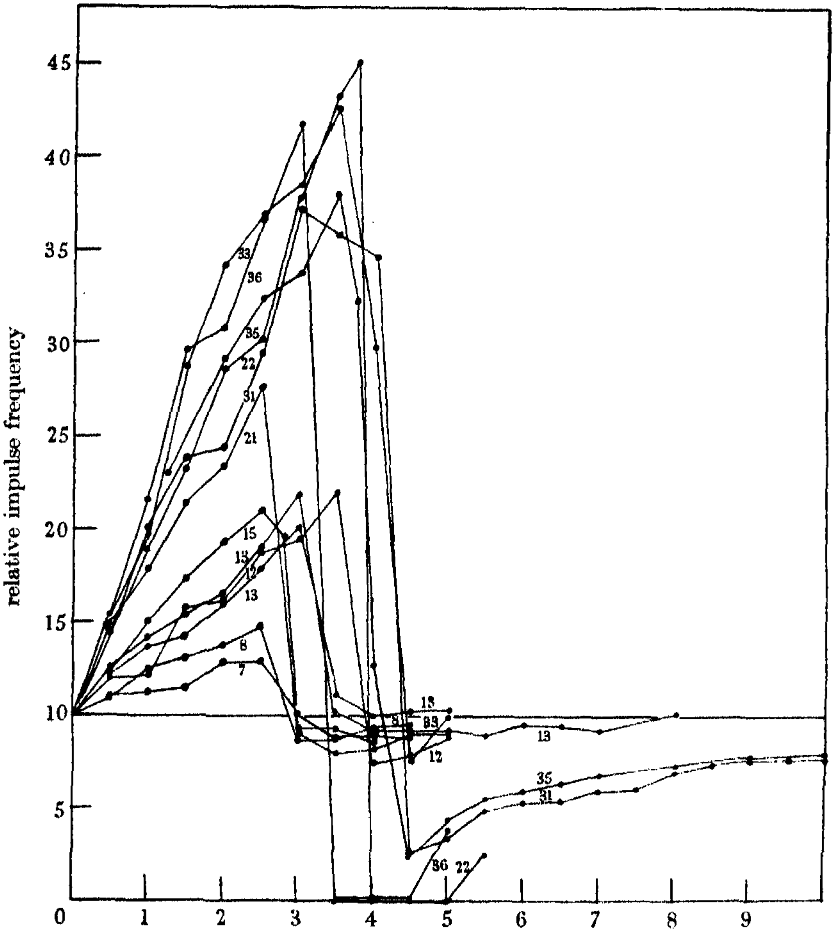

FIGURE 6. Twelve responses of two isolated preparations of the left labyrinth to ipsilateral accelerations ranging from 7 to 36° per sec.^{2}. Duration of acceleration varies between 2·5 and 3·8 sec. In each determination the arrest of rotation coincides with the peak frequency attained. The magnitude of acceleration in degrees per sec.^{2} is marked against each curve.

is the remarkable linearity of the gradual increase or decrease in frequency during uniform acceleration, and this is a conclusion that is confirmed by all our other records. Further, these figures show that quick arrest of an ipsilateral rotation is followed by a negative after-effect, and quick arrest of a contralateral rotation is followed by an after-discharge.

In figures 6 and 7 we have plotted the frequency responses of twelve recordings of ipsilateral rotations at different accelerations, and eleven recordings of contralateral rotations. The resting frequencies on these records ranged between 5·5 and 13 per sec., and, for the sake of clarity, we have corrected all the plotted frequencies in proportion to a resting frequency of 10 per sec. The linearity of the responses is clearly seen, the after-effects are usually present, and the proportionality between the speed of acceleration and the intensity of the response is shown very convincingly by the curves of figure 6, rather less conclusively by figure 7. The range of accelerations applied in figure 6 is from 7 to 36° per sec.^{2}, and from 9 to 63° per sec.^{2} in figure 7. It is worth noting that an acceleration of 7° per sec.^{2} implies a displacement of 180° from rest in 7·2 sec., and an acceleration of 63° per sec.^{2} means 180° displacement in 2·4 sec.

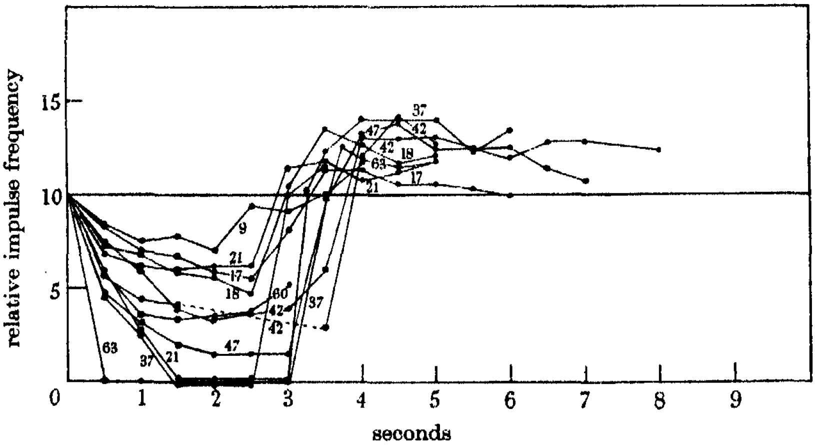

FIGURE 7. Same two preparations as in figure 6. Eleven responses to contralateral accelerations ranging from 9 to 63° per sec.^{2}.

In order to obtain an estimate of the threshold value of the excitatory effect of ipsilateral acceleration we have plotted from the data of figure 6 the percentage increase in frequency after 2·5 sec. of uniform acceleration and extrapolated to zero. This is shown in figure 8. The threshold acceleration appears to lie in the region of 3° per sec.^{2}.

To rotate the turntable at constant speed the falling weight mechanism was disconnected and the drive taken from the high-speed motor. The rotation signal served to test the constancy of the speed of revolution. It was found that, on starting the motor, acceleration was almost instantaneous and the turntable acquired from the very beginning a uniform velocity of great constancy. In figure 9 are shown the responses of a single unit to prolonged ipsilateral and contralateral rotation at 36° per sec. An interval of 10 min. intervened between the two rotations. In this preparation the frequency of the resting discharge was rather higher than usual, being 24–25 a sec. This had the advantage of allowing a greater frequency range for the inhibitory effect of contralateral rotation to manifest itself, and, as the figure shows, the total effects of the two antagonistic rotations were quantitatively the same. The responses in this instance are strikingly different from those observed under constant acceleration. At the beginning of rotation there occurs an abrupt increase or a total inhibition of the frequency of discharge, and this is followed by

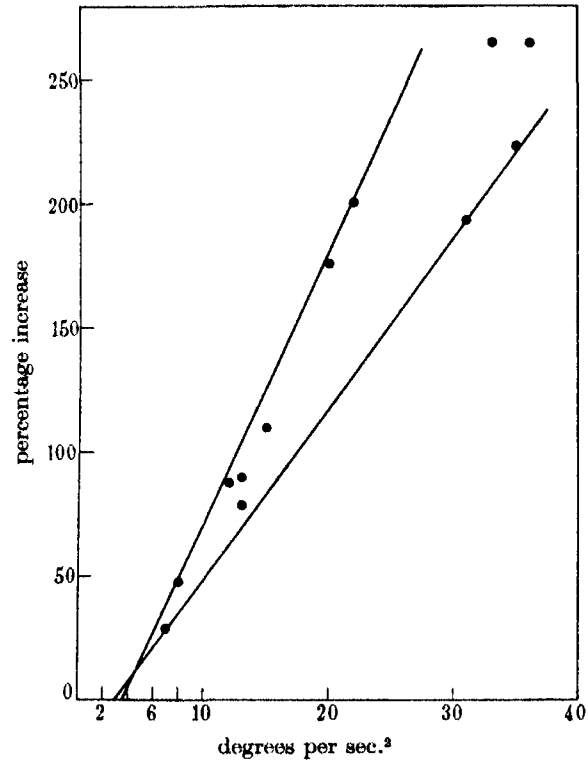

FIGURE 8. Graph showing stimulus-response relation. Data taken from figure 6. The impulse frequency at 2·5 sec. after the beginning of the stimulating acceleration is plotted as percentage increase of the spontaneous frequency.

a slow return to the frequency of the resting discharge. The question at once arises whether this is to be interpreted as an adaptation phenomenon. If it were so interpreted, it would have to be assumed that the deformation of the receptor brought about by the sudden acceleration at the beginning of rotation was indefinitely and steadily maintained throughout the prolonged rotation at constant speed. It is, in fact, a question of whether or not the cupula is an elastic body. On this point the observations of Steinhausen (1933) appear to us to be decisive. He observed the deflexion of the cupula in the pike under conditions almost identical with ours, except

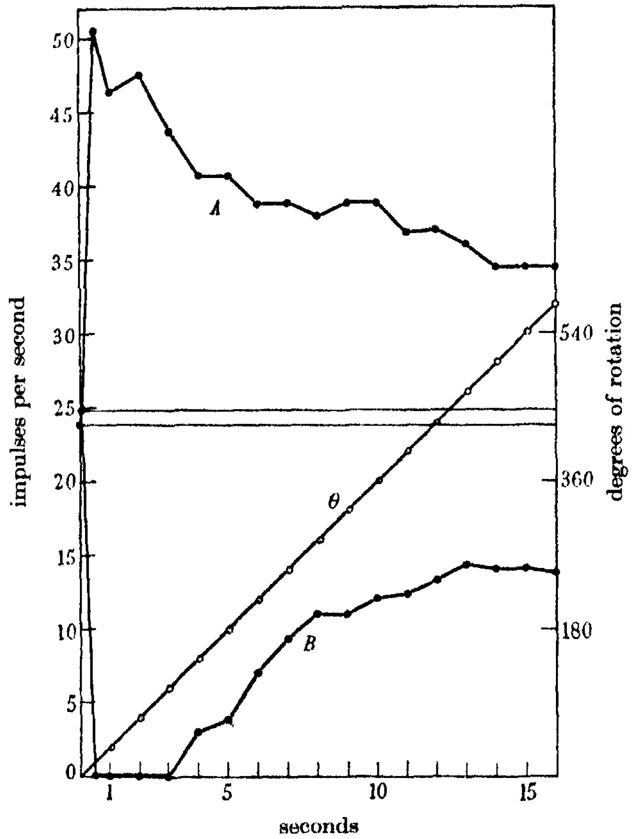

FIGURE 9. Responses of a single receptor unit of the left horizontal canal to rotation at a constant speed of 36° per sec. The two horizontal lines mark the frequencies of the resting discharge before the beginning of each rotation. *A*, response to ipsilateral rotation; *B*, response to contralateral rotation; *θ*, angular displacement of the turntable from rest in either of the two rotations.

that the speed of rotation was five times as great, and gives a graph of the behaviour of the cupula which corresponds fairly closely with our curves of the frequency response. The duration of the return of the cupula to its resting position during sustained constant rotation was 20 sec. It is of interest in this connexion that Ross (1936) described prolonged impulse discharges lasting up to 17 sec. to occur in the eighth nerve of the frog during and after rotary stimulation. It would appear, therefore, that adaptation, if it is a property of the receptors of the semicircular canals at all, plays no significant part in the behaviour of this mechanism. The linearity of the response to a constant *acceleration* adds support to this conclusion. The frequency of the discharge in a single receptor unit must be regarded as being determined primarily, perhaps exclusively, by the degree and direction of deformation, within physiological limits, of the cupula.

CONCLUSIONS

The results of our experiments are in complete agreement with the Mach-Breuer theory of canal mechanism. There is nothing to suggest that the semicircular canal departs in any significant respect from the physical model of an annular tube filled with fluid. But our results advance our understanding in two ways. They define the characteristics of the neural mechanism of the end organ, and they combine with Steinhausen's observations to focus our attention on the cupula and its physical properties.

The neural mechanism has been discussed in previous publications. Its fundamental property is a permanent state of excitation which gives rise to a persistent, spontaneous rhythm of impulse discharge. The origin of this excitation must remain for the present a matter of conjecture. It is possible, on the one hand, that the end organ contains within itself a rhythmical, self-exciting mechanism similar to those which have been suggested to be the cause of central nervous rhythms. On the other hand, it may be supposed that the receptor is maintained in a permanent state of tension—Huizinga's 'gewisse Spannung'—which, owing to the non-adapting quality of the end organ, gives rise to the persistent discharge. It is difficult, in the case of a deformation receptor, to distinguish between these two possibilities, and the distinction probably has little meaning. The essential fact is that the rhythmical excitation is there when no deforming force operates upon the cupula. For it is only by envisaging the cupula as an elastic body which, when released from an imposed deformation returns to its normal position of rest, that the behaviour of the semicircular canal can be interpreted.

It will be, of course, of the utmost importance to determine whether the spontaneous discharge and its inhibition by contralateral rotation occur generally in vertebrates, but the difficulty of recording from an individual ampulla of a bony labyrinth may well prove insuperable. So far the phenomenon has been demonstrated only in elasmobranchs, though the spontaneous discharge was first described by Hoagland (1935) in the lateral-line nerves of teleostean fishes.

In earlier publications (Löwenstein and Sand 1936; Löwenstein 1937) we have argued that the inhibition of the spontaneous discharge by contralateral rotation provides a basis for the occurrence of reflex responses to rotation in both directions in unilaterally operated animals. The quantitative behaviour of single-fibre preparations, as illustrated by figure 9, shows that the magnitude of sensory effect, in terms of frequency change, can be as great during contralateral as during ipsilateral rotation. The eye movements of nystagmus, as Lorente de Nó (1931) has shown, involve not only the contraction of appropriate muscles, but also the inhibition of their antagonists. It may well be that the co-ordination of the activity of the motor centre depends not only on the excitation of the ampulla which is ipsilaterally rotated, but in equal measure on the simultaneous inhibition of the contralateral ampulla.

The importance of the cupula is that, while during angular acceleration it follows the relative movement of the endolymph and is deflected by it, once a uniform angular velocity has been attained the cupula does not, like the endolymph, remain in place relative to the wall of the canal, but by virtue of its elasticity slowly returns to its resting position. It may even be that the cupula, if it fits closely enough into the ampulla, pushes the endolymph back again in returning to rest. There is no evidence on this point, and it is of no great practical importance. When a prolonged rotation is discontinued, the cupula, having returned to rest during the rotation, is deflected in the opposite direction by the deceleration, and from there again slowly returns to rest. Thus the sensory effects of acceleration and deceleration are only equal and opposite if a rotation at constant speed has intervened between them of sufficient duration to permit the cupula to return to rest after its deflection by the acceleration. This duration, according to Steinhausen's data and ours, is of the order of 20–30 sec. If deceleration occurs sooner than 20 sec. after acceleration the deflexion of the cupula in the antagonistic direction is proportionately less, and the sensory after-effect is similarly reduced. These considerations are illustrated graphically in figure 10.

Prolonged rotations at constant speed are only experienced by animals in a laboratory. In nature the displacements with which the labyrinths are mostly called upon to deal are quick acceleration followed immediately by deceleration. Under such conditions the deflexion of the cupula is no more than a monophasic swing, and no sensory after-effects can be expected to follow.

From these considerations it follows inevitably that the duration of nystagmus and after-nystagmus, as well as the dependence of the latter on the duration of rotation at constant speed, are peripherally determined by the physical properties of the cupula. It must be constantly borne in mind that in considering the responses of an intact animal we are dealing with the two cupulae of the two antagonistic horizontal canals. However prolonged the rotation, sensory stimulation continues only during the initial period occupied by the return of the cupulae to rest, and nystagmus can occur only during that period. The sensory after-effect, upon which after-nystagmus depends, can never have a duration longer than the period of the return of the cupulae to rest, and it only has that full duration when the rotation preceding it was at least equally prolonged. The duration of the sensory after-effect, and therefore of after-nystagmus, will depend also upon whether rotation is stopped abruptly or gradually. The

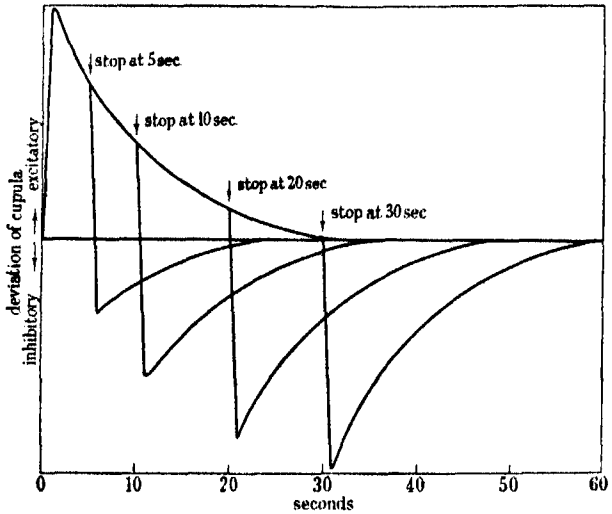

FIGURE 10. Scheme to show deflexion of cupula and change in frequency of impulse discharge during and following prolonged rotation at constant speed. Arrows mark arrest of rotation. The resting position of the cupula and the frequency of the resting discharge correspond to the level of the horizontal line.

quicker the deceleration, the greater will be the deflexion of the cupulae in the opposite direction, and the more prolonged the sensory after-effect and the after-nystagmus.

Rohrer and Masuda (1926), in a theoretical discussion of physical processes in the labyrinth, arrived at similar conclusions. Our observations furnish an experimental verification of their argument.

The cost of the apparatus was defrayed in part by a grant from the Rockefeller Foundation, and in part by a grant from the Government Grants Committee of the Royal Society. Our thanks are due also to Dr O. H. Schmitt for his generous advice and help in the design and construction of the cathode-ray equipment, and to Professor A. V. Hill, F.R.S., for his kind permission to one of us (A. S.) to build the apparatus in his laboratory at University College, London. One of us (O. L.) is greatly indebted to the Physiological Society for the tenure of the Society's table at the Laboratory of the Marine Biological Association during the summer months of 1939. His thanks are also due to the Director and Staff of this Laboratory for their hospitality and help.

SUMMARY

1. Single-fibre discharges have been recorded from the horizontal ampulla of the isolated labyrinth of *Raja*.

2. Responses to different speeds of angular acceleration and to rotation at constant angular velocity are described.

3. The existence of a spontaneous rhythm of discharge which is increased by ipsilateral and inhibited by contralateral rotation is confirmed.

4. During uniform angular *acceleration* the frequency of impulses increases or decreases gradually in a linear manner, and the rate of increase or decrease is proportional to the speed of acceleration.

5. The threshold speed of acceleration is in the region of 3° per sec.^{2}.

6. During prolonged rotation at constant speed the frequency of discharge, having attained a maximum or minimum co-incidentally with the initial acceleration, gradually returns to the spontaneous value over a period of about 20–30 sec.

7. These results are interpreted in terms of the physical properties of the cupula as described by Steinhausen.

8. The spontaneous discharge and its excitation and inhibition by ipsilateral and contralateral rotation provide a basis for the interpretation

of labyrinthine tone, and of the reflex responses to rotation in both directions of unilaterally operated animals.

9. The sensory activity of the semicircular canal, interpreted in terms of the physical properties of the cupula, provides a framework which is adequate to account for the time-relations of nystagmus and after-nystagmus.

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Succinic dehydrogenase-cytochrome system ~~of cells~~

Intracellular respiratory system catalysing aerobic oxidation of succinic acid

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Succinic dehydrogenase-cytochrome system, even in the cell-free colloidal preparation, behaves like a true respiratory system of the cell, showing the high catalytic activity and being affected by all the inhibitors in the same way and to the same degree as the respiration of intact cells. The efficiency of this system depends not only on the integrity of all its components but also on that of the colloidal structure which supports them and assures their mutual accessibility. The paper deals with the properties of each of the components of the system and with the mechanism of their interreactions.

I. Introduction

In this paper we propose to examine the properties of the intracellular system catalysing the aerobic oxidation of succinic acid. The best material for this study is the heart muscle preparation obtained by a method previously described (Keilin and Hartree 1938). This preparation consists of a colloidal suspension of protein particles free from fat, haemoglobin and most of the myosin. It contains the complete cytochrome system, succinic dehydrogenase, fumarase, catalase, flavoprotein compounds and small concentrations of malic, lactic and other dehydrogenases. This preparation, being completely devoid of substrates and of coenzymes, has no oxygen uptake and anaerobically reduces neither cytochrome nor methylene blue. On addition of succinic acid, cytochrome becomes rapidly reduced and in presence of air the preparation now shows a rapid oxygen uptake. In this preparation succinic acid, activated by succinic dehydrogenase, is oxidized aerobically by means of the cytochrome system which can be seen to undergo rapid oxidations and reductions.

The system catalysing the aerobic oxidation of succinic acid is of special interest as it represents so far the only known complex intracellular oxidation system which in a cell-free preparation behaves exactly as the natural respiratory system of the cell. In fact, this system shows a very great catalytic activity ($Q_{O_2} > 500$) without addition of artificial non-biological carriers or fixatives. It has a great affinity for its substrate (succinic acid) and its activity is affected by all inhibitors in the same way and to the same degree as the normal respiration of intact cells. Moreover, the activity of this system depends not only on the properties of its individual components but also on those of the colloidal protein particles to which they are more or less intimately bound. It is conceivable that each of the colloidal particles acts as a support for the complete system and thus assures the mutual accessibility of its components.

Of all the components of the system involved in the oxidation of succinic acid, so far only cytochrome *c* has been extracted in a pure state. This does not mean that *c* is less intimately bound to the colloidal particles than are the other components of the system, but, being very stable, it can withstand all the treatment necessary for its isolation and purification. The other components of the system, being very labile, have as yet not been isolated in an unmodified and active state and their properties can therefore be studied only in an intact preparation containing several other catalytic systems.

It is important to note that in higher organisms a typical and well developed cytochrome system with its characteristic absorption spectrum is found mainly in skeletal muscles, in heart muscles and in the grey matter of the brain. In cells of most other tissues such as kidney, liver, pancreas, etc., cytochrome is somewhat modified. Spectroscopically the band *a* is comparatively feeble and the bands *b* and *c* are replaced by a broad band b_1. The properties of this modified cytochrome will be examined in the chapter dealing with the activity of the complete system in kidney preparations (p. 283).

II. PROPERTIES OF CYTOCHROME *c*

Cytochrome *c* used in this investigation was prepared by the method previously described (Keilin and Hartree 1937). Of its properties we enumerate here only those which have a direct bearing on our problem:

(1) Within the physiological range of pH reduced cytochrome *c* is not autoxidizable and does not combine with CO.

(2) It becomes autoxidizable at pH below 4 or above 12 and at about 13 combines reversibly with CO.

(3) Biologically cytochrome *c* is oxidized by cytochrome oxidase and can be reduced by the succinic dehydrogenase system and by a variety of substances such as cysteine, ascorbic acid, *p*-phenylenediamine and hydroquinone which do not require specific activation.

(4) Although cytochrome *c* is a conjugated protein it shows a remarkable thermostability. On boiling a solution of *c* its colour turns from red to brown and the bands of parahaematin disappear; on cooling, the colour, the absorption spectrum and all other properties return to the original state.

(5) Cytochrome *c* withstands prolonged treatment with strong acids which modify its parahaematin absorption spectrum to that of acid haematin. A solution of *c* kept for 18 hr. in 0·3 N HCl reverts after neutralization and dialysis to its normal colour, absorption spectrum, behaviour towards O_2 and CO and catalytic properties.

(6) It also resists treatment with alkali. A solution of *c* treated with 0·1 N KOH for 18 hr. at 20° C. is not completely modified. On neutralization at least 50 % of the compound recovers its original properties. However, on treating cytochrome *c* in a similar way with N KOH, the pigment after neutralization and dialysis does not revert to its original state. Although it still shows an absorption spectrum of the cytochrome *c* type, its bands are slightly shifted towards the red end of the spectrum. Cytochrome *c* thus obtained is completely autoxidizable and forms with CO a light-sensitive compound. This compound will be referred to in this paper as autoxidizable *c*.

III. ATTEMPTS AT RECONSTRUCTION OF THE COMPLETE SYSTEM

Attempts to split the succinic-cytochrome system into its components in order to reconstruct from them the complete system have so far failed. The claims by some workers of having isolated from cells or tissues components other than *c*, or of having successfully reconstructed the complete system are not supported by proper experimental evidence and cannot therefore be accepted. This can be clearly seen from a brief examination of the following three examples of such claims:

(A) It was stated by Euler and Hellström (1939) that tissue preparations can be separated into several fractions such as (1) succinic dehydrogenase, (2) cytochrome *c*, (3) cytochrome *b*, (4) cytochrome *a* + *b* and (5) cytochrome oxidase from which the complete succinic dehydrogenase-cytochrome system can be reconstructed. Careful examination of their manometric results shows, however, that the first, fourth and fifth fractions already contain a more or less complete system. It is, moreover, surprising that the activity of the mixture devoid of the fractions containing the component *a* and cytochrome oxidase instead of being completely inactive is only 30 % below the activity of the complete system.

(B) By ultracentrifuging a heart muscle preparation obtained by our method, Stern and Melnick (1939) claim to have separated it into two aerobically inactive fractions which on recombination form a complete system catalysing the aerobic oxidation of succinic acid. This result is difficult to reconcile with the fact that the colloidal preparation of heart muscle can be washed several times in buffer solutions of pH up to 8 in an ordinary rapid centrifuge (13,000 r.p.m.) without great loss of activity. Yet according to these workers such washing should completely inactivate the sediment as the supernatant fluid obtained under these conditions should contain the whole of the fraction which has failed to sediment in their experiments.

(C) The claim by Altschul, Abrams and Hogness (1939) to have isolated from bakers' yeast a soluble cytochrome oxidase cannot be accepted for several reasons:

(*a*) The activity of their 'enzyme' preparation expressed in terms of Q_{O_2} is only 152 at 25° C which is extremely low compared with the respiratory activity of intact yeast cells which at 20° have $Q_{O_2} = 100$. We know, on the other hand, that the activity of cytochrome oxidase even in yeast cells is far from being the limiting factor in their respiration. Moreover, the Q_{O_2} of cytochrome oxidase in our heart muscle preparation may be as high as 1400 at 39° C although in addition to cytochrome oxidase the preparation contains all the components of cytochrome, succinic dehydrogenase, catalase, fumarase, small concentrations of other enzymes, a certain amount of myosin and other proteins.

(*b*) Their enzyme preparation is strongly inhibited by catalase while the cytochrome system in yeast and especially in muscle preparations is not affected even by a very great excess of catalase.

(*c*) Their enzyme does not seem to be affected by alcohol which rapidly destroys cytochrome oxidase in yeast and muscle preparations.

It is, however, impossible to discuss the nature and function of their enzyme preparation until its activity is tested manometrically in a system composed of the enzyme, cytochrome *c* and a substance such as *p*-phenylenediamine, its absorption spectrum is determined and the metal (if any) in its active group is identified.

It was shown previously that oxidized soluble *c* added to the enzyme preparation containing succinic acid undergoes immediate reduction (Keilin 1929) which indicates that soluble *c* can act as a carrier between succinic dehydrogenase and cytochrome oxidase.

It was also shown that the addition of a very small amount of soluble *c* to the enzyme preparation greatly increases its catalytic activity towards such substances as cysteine (Keilin 1930), succinic acid (Ogston and Green 1935), *p*-phenylenediamine, ascorbic acid, hydroquinone and adrenaline (Keilin and Hartree 1938). The increase in the catalytic activity of the enzyme preparation on addition of *c* indicates that either the *c* present in the preparation does not exhibit its full activity or that the preparation is more or less deficient in this component. In fact both these conditions may be responsible for the low catalytic activity of some enzyme preparations which will now be described.

IV. REACTIVATION BY SOLUBLE *c* OF HEART MUSCLE PREPARATIONS CONTAINING FIXED *c*

Considering that cytochrome *c* is a very stable compound it is unlikely that it can be affected in any way by the simple manipulations of preparation of the enzyme.

The comparatively low activity of *c* in some preparations can be ascribed only to its fixed position in the protein aggregates and to its lack of accessibility to the other components of the system. This is strongly supported by the fact that the activity of a preparation can be greatly reduced by a treatment which does not affect the properties of *c* and yet can be restored by the addition of soluble *c*. The best example of such treatment is repeated freezing of the preparation in liquid air. For this purpose a fresh preparation of pig heart muscle obtained in the usual way is three times frozen in liquid air followed by thawing at room temperature. After this treatment the preparation seems only to be more aggregated while the absorption spectrum of cytochrome is unchanged.

The effect of this treatment on the rate of oxidation of *p*-phenylenediamine and of succinic acid with and without addition of cytochrome *c* is shown in table 1.

TABLE 1

	cu. mm. O_2 uptake in 5 min.		
Systems	Untreated	After freezing	% inhibition due to freezing
Enz. + *p*-ph.-d.	114	18	84
Enz. + *p*-ph.-d. + *c*	208	186	10
Enz. + succ.	96	23	76
Enz. + succ. + *c*	134	131	2

Enz., enzyme preparation: 0·1 c.c. for oxidation of *p*-phenylenediamine (*p*-ph.-d.) and 0·2 c.c. for oxidation of succinic acid. Cytochrome *c* is added after freezing.

These experiments clearly demonstrate that freezing and thawing strongly inhibits the catalytic activity of the heart muscle preparation and that this inhibition is almost completely abolished by the addition of a small amount of *c*. In other preparations treated by freezing, the ratio between the rates of oxidation of succinic acid in presence and in absence of *c* has reached even higher values as shown in table 2 and figure 1.

All manometric experiments described in this paper were carried out at 39° C and pH 7·3 (0·1 M phosphate buffer) in Barcroft manometers. Unless otherwise stated the concentrations of the ingredients were as follows: *p*-phenylenediamine 0·05 M; sodium succinate 0·02 M; cytochrome *c* (excess) 6×10^{-5} M; KCN 0·01 M; methylene blue 0·001 M; Enz. 0·1–0·2 c.c. (2–4 mg. dry weight) heart muscle preparation; and 0·5 c.c. kidney preparation.

TABLE 2

Systems	Q_{O_2}
Enz. + *p*-ph.-d.	42
Enz. + *p*-ph.-d. + *c*	434
Enz. + succ.	11
Enz. + succ. + *c*	306
Enz. + succ. + M.B.	157

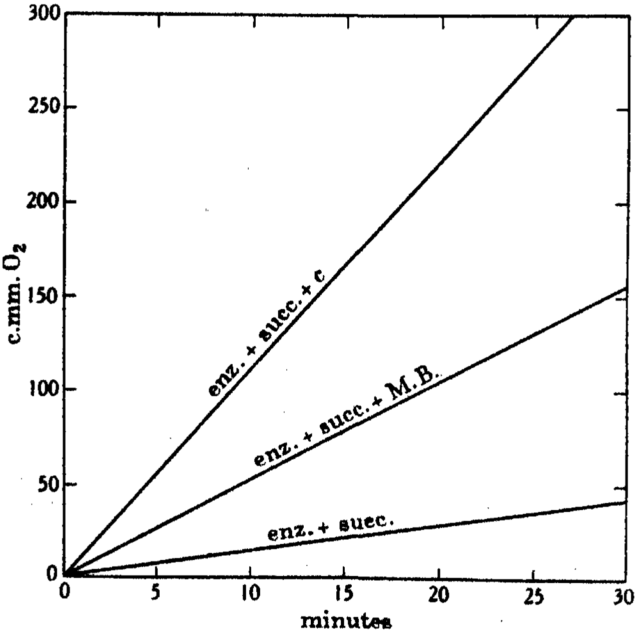


FIGURE 1. Effect of cytochrome *c* (*c*) and methylene blue (M.B.) on oxidation of succinic acid (*succ.*) by heart muscle preparation (*enz.*) treated previously with liquid air.

The fact that freezing does not affect any components of the succinic dehydrogenase-cytochrome system suggests that its effect is caused only by a diminution in the mutual accessibility of these components. These experiments also indicate that the great efficiency of catalytic systems within living intact cells is due to a high structural organization of cells responsible for the proper spatial distribution and the most favourable molecular orientation of all components of the system.

V. REACTIVATION BY SOLUBLE *c* OF KIDNEY PREPARATION DEFICIENT IN *c*

That the tissue from the cortical layer of pig kidney is deficient in cytochrome *c* is shown by the fact that the oxidation of succinic acid by unwashed minced tissue or after grinding the tissue with sand is distinctly increased by the addition of soluble *c* (table 3). The effect is, however, more marked in a kidney preparation obtained as follows:

The cortical layer of one pig's kidney is passed through a Latapie mincer, stirred for about 10 min. in 1 l. water and pressed out through muslin. The remaining pulp is stirred with a further 1 l. water and pressed out. The combined fluids are centrifuged, the precipitate discarded while the cloudy reddish fluid is treated with the minimum of acetic acid to cause precipitation (pH 5·5). The precipitate is centrifuged off, washed with 0·02 M KH_2PO_4 and suspended in an equal volume of 0·1 M phosphate buffer pH 7·3.

TABLE 3

	Q_{O_2}			
	+ succ.		+ *p*.-ph.-d.	
Kidney preparations	− *c*	+ *c*	− *c*	+ *c*
Mince unwashed	32	50	27	94
Ground unwashed	20	45	14	77
Enzyme preparation	11	132	12	214

This preparation always contains traces of oxyhaemoglobin. On reduction with $Na_2S_2O_4$ it shows a very narrow absorption band *a* at about 602 mμ and a wide, dark and asymmetrical band b_1 at about 555 mμ. This wide band seems to be composed of two very narrow bands of unequal intensity united by a dark shading. The geminated structure of this band can be clearly seen in a preparation cooled to the temperature of liquid air. Under this condition the bands *a* and b_1 are greatly intensified and b_1 is split into two bands: a stronger band at about 558 mμ and a feeble band at 551 mμ. It is, however, impossible to say whether these two bands represent the two absorption maxima of one compound or belong to two distinct compounds. The band at 551 mμ does not belong to *c* which is apparently missing in this preparation. In fact the α-band of cytochrome *c* at the temperature of liquid air is shifted from 549·5 to about 547 mμ.

On addition of succinic acid to kidney preparation the oxidized components of its cytochrome are reduced very slowly. If, however, a small amount of *c* is added it becomes rapidly reduced and this reduction is accompanied by that of *a* and of b_1. Spectroscopic study shows, therefore, that the succinic system which is very inactive in this preparation can be brought into activity by the addition of cytochrome *c*. These observations are confirmed by manometric experiments summarized in figure 2 and table 3.

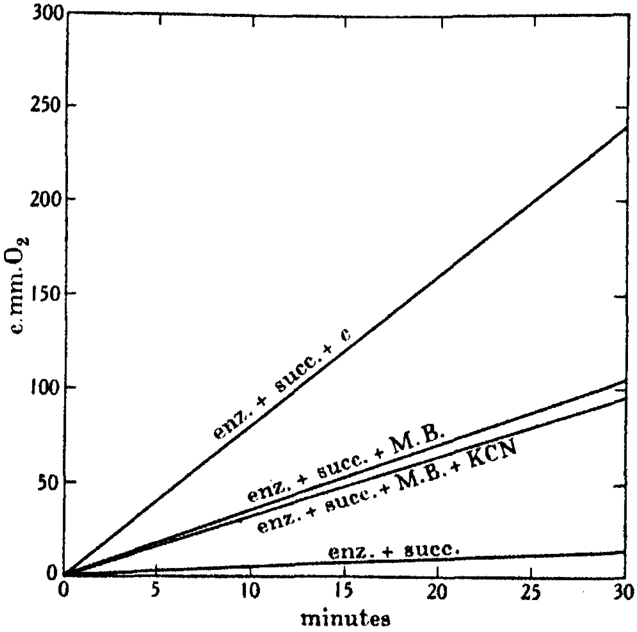


FIGURE 2. Experiment similar to that shown in figure 1 except that enzyme preparation was obtained from the cortical layer of kidney.

The comparatively slow oxidation of succinic acid and of *p*-phenylenediamine by minced unwashed kidney tissue without addition of *c* can be explained in two ways: either the tissue contains a small amount of *c* which is easily removed by washing, or component b_1 in these reactions replaces *c* but is not as efficient as *c*.

The comparative study of preparations obtained from heart muscle and from kidney shows that the maximum activities of the cytochrome system expressed in terms of Q_{O_2} for *p*-phenylenediamine in presence of an excess of *c* is proportional to the intensities of the band *a* in these two preparations.

VI. ACTIVITY OR 'TURN OVER NUMBER' OF CYTOCHROME *c*

The real catalytic activity of cytochrome *c* can be easily determined by adjusting the concentrations of enzyme and of *c* so as to attain a maximum increase in the oxygen uptake with the minimum amount of *c* added. The results of such experiments show that the addition of $2{\cdot}15 \times 10^{-9}$ g. atom Fe of *c* (which is equivalent to 0·012 cu.mm. O_2) to a heart muscle preparation containing succinic acid increases the oxygen uptake of the system during the first 10 min. by 170 cu. mm. The turnover number for cytochrome *c*, which is the ratio of the O_2 uptake per minute to the O_2 equivalent of cytochrome *c* added, is therefore about 1420. This is obviously a very high value considering that the activity of cytochrome *c* in this experiment was estimated on a reconstructed system composed of several components, the mutual accessibility of which could hardly be as efficient as in living cells. It represents, moreover, about one-third of the estimated value (3850) for the turnover of cytochrome *c* in living yeast cells. The turnover in yeast was determined in the following way:

A suspension of bakers' yeast was prepared containing 118 mg. dry weight per c.c. A sample of this suspension was heated to 80° C for 20 min., which destroys all the components of cytochrome except *c*. On reduction, this suspension shows only the absorption bands of *c* which clearly stand out owing to the absence of other bands. The intensity of the α-band of *c* in the yeast suspension was matched spectroscopically with that of a standard solution of pure *c* and was found to contain $1{\cdot}83 \times 10^{-8}$ g. atom of Fe of *c* per c.c. (which is equivalent to 0·102 cu. mm. O_2). As the Q_{O_2} of this suspension at 38° C was 200, the oxygen uptake of 1 c.c. of this suspension per min. will be $\frac{118 \times 200}{60}$ and the turnover of $c = \frac{118 \times 200}{60 \times 0{\cdot}102} = 3850$. It may be interesting to note that 1 mg. of dry weight of yeast contains $2{\cdot}6\,\mu$g. of cytochrome *c*, i.e. 0·26 %. The Q_{O_2} of cytochrome *c* during the active respiration of yeast is therefore about 80,000.

VII. CATALYTIC PROPERTIES OF AUTOXIDIZABLE AND NON-AUTOXIDIZABLE CYTOCHROME *c*

It was shown above (p. 279) that a prolonged treatment with strong alkali irreversibly modifies cytochrome *c* which becomes autoxidizable and combines with CO. It is interesting to examine to what extent this treatment affects its catalytic properties. For this purpose the activities of the modified *c* and of unmodified *c* were tested manometrically in the systems oxidizing *p*-phenylenediamine and succinic acid.

The experiments recorded in table 4 and figures 3 and 4 clearly demonstrate that when cytochrome *c* becomes autoxidizable and capable of combining with CO it loses completely its catalytic properties. This is mainly due to the fact that neither the succinic system nor *p*-phenylenediamine can reduce it with sufficient velocity.

TABLE 4

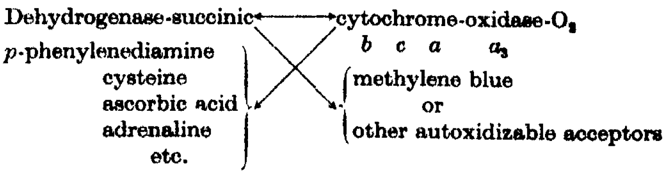
Systems	cu. mm. O_2 uptake in 20 min. Prep. I	Prep. II
Enz. + *p*-ph.-d.	30	27
Enz. + *p*-ph.-d. + autox. *c*	35	32
Enz. + *p*-ph.-d. + non-autox. *c*	370	300
Enz. + succ.	20	30
Enz. + succ. + autox. *c*	20	30
Enz. + succ. + non-autox. *c*	230	260

More concentrated enzyme preparation was used for oxidation of succinic acid (as in table 1). Enz., enzyme preparation treated with liquid air as previously described.

The non-autoxidizability of cytochrome *c* is therefore a property intimately connected with its catalytic activity and in this respect cytochrome *c* resembles other intracellular catalysts such as coenzymes and the biologically active flavoproteins.

VIII. RELATIVE ACTIVITIES OF SUCCINIC DEHYDROGENASE AND CYTOCHROME SYSTEMS

The system oxidizing succinic acid as we have seen previously is composed of two distinct portions, the activities of which can be determined independently:



The activity of succinic dehydrogenase can be determined independently of the cytochrome system either anaerobically from the rate of reduction

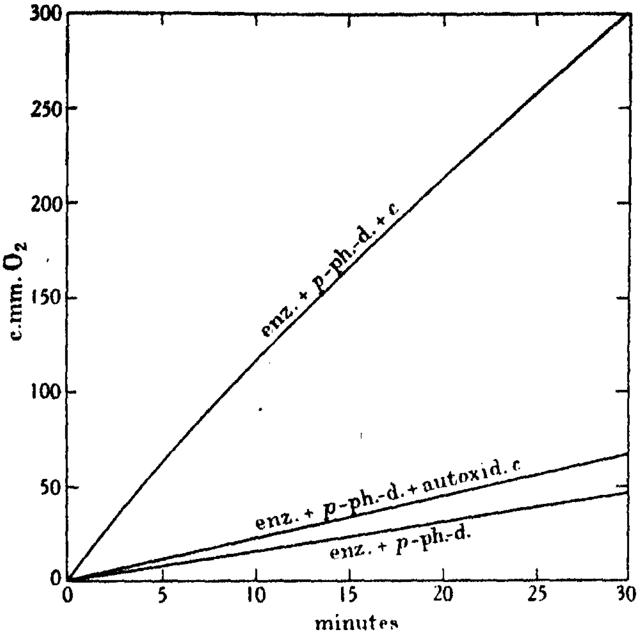


FIGURE 3. Effect of autoxidizable (autoxid. *c*) and non-autoxidizable (*c*) cytochrome c on oxidation of *p*-phenylenediamine by heart muscle preparation (treated previously with liquid air).

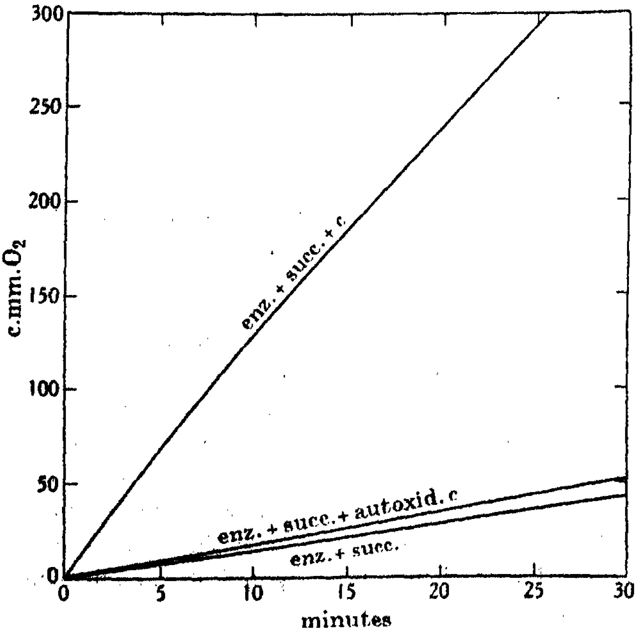


FIGURE 4. Effect of autoxidizable (autoxid. *c*) and non-autoxidizable (*c*) cytochrome c on oxidation of succinic acid by heart muscle preparation (treated previously with liquid air).

of methylene blue (M.B.) or aerobically from the rate of oxygen uptake in presence of M.B. and cyanide which inactivates the cytochrome system.

The activity of the cytochrome system can be determined from the rate of oxidation of *p*-phenylenediamine or other substances not requiring activation by dehydrogenases. In all tests where the cytochrome system is involved it is essential to supplement the system with an excess of soluble cytochrome *c*.

The activities of the two portions of the succinic dehydrogenase-cytochrome system tested separately and in a complete system are shown in table 5. This experiment shows that the activity of cytochrome when tested with *p*-phenylenediamine (1) is about double its activity when tested in a complete system with succinic acid (3). This indicates that the activity of the cytochrome system in our preparation is about twice as high as that of the succinic dehydrogenase system. From this experiment we can see also that the oxidation of succinic acid by the cytochrome system (3) is much more efficient than by methylene blue (5) (figure 1). This is, moreover, confirmed by the study of kidney preparations (figure 2). Other experiments have revealed that while the turnover of cytochrome *c* in the system oxidizing succinic acid may be as high as 1400 the turnover of methylene blue is only 2–3 under optimum conditions.

TABLE 5

	Q_{O_2}
1. Enz. + *c* + *p*-ph.-d.	930
2. Enz. + *c* + *p*-ph.-d. + KCN	0
3. Enz. + *c* + succ.	560
4. Enz. ± *c* + succ. + KCN	0
5. Enz. ± *c* + succ. + KCN + M.B.	310

IX. EFFECT OF DIFFERENT FACTORS ON SUCCINIC DEHYDROGENASE AND ON THE CYTOCHROME SYSTEM

(1) *Effect of* KCN, H_2S *and* NaN_3

These substances in small concentration inhibit reversibly the activity of cytochrome oxidase (Keilin 1929). They inhibit the aerobic oxidation of succinic acid and of substances not requiring activation, such as *p*-phenylenediamine, while they have no effect on the oxidation of succinic acid by methylene blue. These three inhibitors, as was recently shown (Keilin and Hartree 1939), combine reversibly with the component a_3 which was identified with cytochrome oxidase. While these inhibitors stabilize the component a_3 in the trivalent state they inhibit the oxidation of the components *a*, *b* and *c*.

(2) *Effect of narcotics*

Narcotics, as shown in table 6, have a much stronger inhibitory effect on the oxidation of succinic acid by the cytochrome system than by methylene blue. Their effect is still less marked on the oxidation of *p*-phenylenediamine which does not require activation by a specific dehydrogenase.

TABLE 6

	cu. mm. O_2 uptake in 10 min.		
Systems	Control	+ 0·1 % phenylurethane	% inhibition
Enz. + *c* + *p*-ph.-d.	380	276	28
Enz. + *c* + succ.	208	52	75
Enz. + *c* + succ. + KCN + M.B.	100	60	40

It is important to remember that while narcotics inhibit the reduction of the component a_3, *a* and *c* they inhibit on the contrary the oxidation of *b*. This indicates that narcotics affect the accessibility of *b* to the rest of the cytochrome system and that this component while essential to the oxidation of succinic acid does not play an important part in the oxidation of substances not requiring activation by dehydrogenases.

(3) *Effect of treatment with alcohol*

20 c.c. of normal preparation is mixed with 20 c.c. 90 % alcohol and left for 10 min. at 20° C. The mixture is then poured into 160 c.c. 0·02 M acid phosphate and centrifuged. The sediment is washed with water, suspended in 16 c.c. 0·1 M phosphate buffer pH 7·3 and dialysed for 3 hr. against 500 c.c. 0·1 M phosphate buffer pH 7·3.

On spectroscopic examination in presence of $Na_2S_2O_4$ this preparation shows the bands *b* and *c* of cytochrome almost fused and band *a* shifted from its usual position (603 mμ) to 583 mμ. Moreover, components *a* and *b* are now autoxidizable and combine with CO.

On addition of succinic acid this preparation reduces neither its own nor added cytochrome *c*.

The catalytic activity of such preparations was tested manometrically in the usual way and the results of these tests are given in table 7.

These experiments clearly demonstrate that the treatment with alcohol completely abolishes the catalytic activity of the cytochrome system by modifying irreversibly the components a_3, *a* and *b*. It also reduces the activity of the dehydrogenase system by about two-thirds, the remaining activity being still fairly high (e.g. about $Q_{O_2} = 100$).

It is important to note that the alcohol treated preparations, even in presence of succinic acid, will not reduce cytochrome *c*.

TABLE 7

Exp.	Catalytic systems	Q_{O_2}
	Untreated enzyme preparation	
1	Enz. + *p*-ph.-d. + *c*	930
	Enz. + succ. + *c*	560
	Enz. + succ. + *c* + KCN	0
	Enz. + succ. + *c* + M.B. + KCN	310
	Alcohol treated enzyme preparations	
2	Enz. + *p*-ph.-d. + *c*	2
	Enz. + succ. + *c*	3
	Enz. + succ. + *c* + M.B.	100
3	Enz. + succ. + *c*	0
	Enz. + succ. + *c* + M.B.	103

To this category of preparations belongs also the enzyme preparation obtained by Hopkins, Lutwak-Mann and Morgan (1939) from heart muscle minced, washed with lithium chloride, extracted with bile salt, precipitated and washed with alcohol, suspended in urea, dialysed and resuspended in buffer solution. Although this treatment destroys completely the labile components of cytochrome and the preparation does not reduce cytochrome *c* in presence of succinic acid it is still capable of oxidizing succinic acid through methylene blue. The Q_{O_2} of this oxidation, however, dropped to about 12 owing to destruction of the main portion of the succinic dehydrogenase itself.

(4) *Effect of treatment with acid*

A sample of heart muscle preparation is acidified with N acetic acid to pH 5·0 and after 1 hr. at room temperature neutralized with ammonia to pH 7·3. The catalytic activity of this preparation (table 8) shows that this treatment hardly affects the cytochrome system involved in the oxidation

TABLE 8

	cu. mm. O_2 uptake in	
	10 min.	20 min.
Enz. + *p*-ph.-d. + *c*	521	857
Enz. + succ. + *c*	3	7
Enz. + succ. + M.B.	114	208

of *p*-phenylenediamine but abolishes almost completely the oxidation of succinic acid in the presence of an excess of cytochrome *c*. On the other hand, the preparation is still able to oxidize succinic acid in the presence of methylene blue.

Spectroscopic examination of this preparation treated with $Na_2S_2O_4$ shows a normal absorption spectrum of reduced cytochrome with only a slight shading between the bands *b* and *c*. On shaking the reduced preparation with air the components *a* and *c* undergo oxidation while *b* remains reduced. This preparation, in the presence of succinic acid, reduces neither the cytochrome *c* which it contains nor added *c*.

(5) *Effect of treatment with pancreatin*

A heart muscle preparation treated for 16 hr. at 17° C with 0·5% pancreatin at pH 8·6 and tested in the usual way gave the results shown in table 9.

TABLE 9

	Q_{O_2}
Enz. + *p*-ph.-d. + *c*	400
Enz. + succ. + c	0
Enz. + succ. + M.B.	138

Here again, although the cytochrome system still remains very active, the preparation is unable to oxidize succinic acid in presence of *c* but oxidizes it well in presence of methylene blue.

Spectroscopic study of this preparation reveals the same properties as in preparations treated with acid, namely, that (1) the oxidation of component *b* is very slow and (2) the preparation has lost its property of reducing cytochrome *c* in presence of succinic acid.

(6) *Effect of treatment with alkali* (*pH* 9)

An enzyme preparation kept one hour at pH 9 and neutralized to pH 7·3 shows a marked change in its properties. The activity of the cytochrome system is reduced by about 30% while the activity of succinic dehydrogenase is almost completely abolished (table 10). This preparation, even in presence of succinic acid, does not reduce cytochrome although no apparent change can be noticed in its absorption spectrum.

The inhibitory effect is much less marked when the preparation is brought to pH 9 in presence of succinic acid. As shown in table 11 succinic acid and to some extent, anaerobic conditions and pyrophosphate protect the succinic dehydrogenase from destruction by this treatment. The pyrophosphate was removed by dialysis before testing.

These experiments suggest that the destruction of succinic dehydrogenase at pH 9 may be due to the oxidation of some groupings the integrity *of which is essential for normal enzyme activity.* That these groupings probably act as links uniting the enzyme to its substrate is shown by the strong protection exerted by succinic acid and the smaller protection by pyrophosphate (a specific inhibitor of the dehydrogenase) against the destructive effect of alkali (see also p. 295).

TABLE 10

	Q_{O_2}	
	Untreated	Treated
Enz. + *p*-ph.-d. + *c*	920	639
Enz. + succ. + *c*	368	7
Enz. + succ. + *c* + KCN + M.B.	205	6

TABLE 11

	cu. mm. O_2 uptake in 20 min.	% activity
Pig heart		
Enz. (untreated) + *c* + succ.	534	100
Enz. (pH 9·0) + *c* + succ. ± M.B.	7	1·3
Enz. (pH 9·0 anaer.) + *c* + succ.	54	10
Enz. (pH 9·0 succ.) + *c* + succ.	349	65
Horse heart		
Enz. (untreated) + *c* + succ.	764	100
Enz. (pH 9·0) + *c* + succ.	28	4
Enz. (pH 9·0 succ.) + *c* + succ.	678	88
Enz. (pH 9·0 pyrophosphate) + *c* + succ.	171	22

Enzyme preparations incubated 1 hr. at pH 9·0 (as above) in presence of 0·02 M succinate and 0·02 M pyrophosphate: 0·3 c.c. taken per manometer.

X. MECHANISM OF INTERACTION BETWEEN THE SUCCINIC DEHYDROGENASE AND CYTOCHROME SYSTEMS

The experiments related in the previous section clearly show that according to the method of treatment of tissue suspensions several enzyme preparations can be obtained exhibiting marked differences in their properties. These enzyme preparations can be classified in the following way:

(1) Preparations which oxidize succinic acid aerobically in presence of *c* and anaerobically in presence of methylene blue. These preparations invariably reduce cytochrome *c* in presence of succinic acid and by means

of *c* oxidize *p*-phenylenediamine. In other words they show the succinic dehydrogenase and cytochrome systems intact and properly linked. All these properties are found in our usual heart muscle preparation.

(2) Preparations which do not oxidize succinic acid aerobically even in presence of *c* but oxidize it anaerobically in presence of methylene blue. To this category belong three different kinds of preparations namely:

(*a*) Preparations obtained by treatment with specific inhibitors (KCN, H_2S, NaN_3); they reduce cytochrome *c* in presence of succinic acid but are unable to oxidize reduced *c*, and do not oxidize *p*-phenylenediamine. The action of these substances is perfectly reversible.

(*b*) Preparations obtained by treatment with acids or pancreatin; they do not reduce *c* in presence of succinate but are capable of oxidizing reduced *c* and also *p*-phenylenediamine in presence of *c*.

(*c*) Preparations obtained by treatment with alcohol; they are incapable of either reducing *c* in presence of succinate or oxidizing reduced *c*. Their cytochrome system is irreversibly modified and naturally they do not oxidize *p*-phenylenediamine. To this group belongs also the preparation obtained by Hopkins, Lutwak-Mann and Morgan (1939).

(3) Preparations which do not oxidize succinic acid in presence of either *c* or methylene blue but oxidize *p*-phenylenediamine in presence of *c*. This result can be obtained by raising the pH of the preparation to pH 9·0 and neutralizing it after 1 hr. to pH 7·3.

Of the preparations discussed above those which are treated with acid or pancreatin are of special interest. In presence of *c* they oxidize *p*-phenylenediamine but not succinic acid, which is, however, oxidized through methylene blue, and in presence of succinic acid they do not reduce cytochrome *c*. Although both portions of the system, cytochrome oxidase and succinic dehydrogenase are present in these preparations they do not inter-react to form a complete system catalysing the aerobic oxidation of succinic acid. A similar preparation composed of both portions of the system and yet incapable of catalysing the aerobic oxidation of succinic acid can be also obtained by mixing the alcohol-treated and the alkaline-treated preparations containing respectively the succinic dehydrogenase and the cytochrome systems.

The fact that preparations can be obtained in which the succinic system does not reduce *c* although it reduces methylene blue indicates that either the reduction of *c* takes place through an intermediary link destroyed in these preparations, or that the succinic system is accessible to methylene blue but not to *c*.

If there is a substance acting as a link between the succinic system and *c* it must be more fragile than a and a_3 of cytochrome. Such a link may be cytochrome b itself which is very labile and is always affected in some way in preparations not reacting with c. This does not exclude, however, the possibility of the existence of yet another labile link which may be either a haematin or a flavin compound. In fact, the intense absorption band which appears between b and c on treating the preparation with pyridine and reducer belongs probably to a pyridine haemochromogen of b mixed with that of another haematin compound the absorption spectrum of which is invisible in an untreated preparation.

On the other hand, the tissue preparations show also two absorption bands (495 and 455 mμ) of a flavoprotein compound which on addition of succinic acid fade simultaneously with the appearance of the bands of reduced cytochrome (Keilin and Hartree 1939). The disappearance of these bands may be either an apparent one and due to changes in the background during the reduction of cytochrome or it may be real and due to the reduction of the flavoprotein itself. In the latter case the flavoprotein would act as one of the components of the system oxidizing succinic acid and belonging either to succinic dehydrogenase itself or to one of the links uniting it with the cytochrome system.

The fact that the succinic system in some preparations does not react with cytochrome while it still reacts with methylene blue can be also explained without postulating the necessary loss or destruction of one of the components of the system. It may be due to an irreversible change in the colloidal structure of the preparation accompanied by a loss of accessibility of the succinic system to c which is a non-diffusible protein while it remains still accessible to small and diffusible molecules of methylene blue. It is of interest to note in this connexion that while a crude lactic dehydrogenase of yeast oxidizes lactic acid through cytochrome and cytochrome oxidase a partially purified one will do so in presence of M.B. but not in presence of c and a strong cytochrome oxidase (Dixon and Zerfas 1939).

We can say in conclusion that so far there is no definite evidence supporting either of these possibilities and this interesting problem may be considered as still open for investigation.

XI. EFFECT OF CERTAIN INHIBITORS ON THE OXIDATION OF SUCCINIC ACID BY TISSUE PREPARATIONS

(1) *Summary of previous results*

We shall examine now the effect of some substances which reversibly inhibit the oxidation of succinic acid in presence of either the cytochrome system or methylene blue. Thus, the inhibitory effects of many substances including copper, fluoride and iodide have been demonstrated by Quastel and Wooldridge (1927). They have shown also that succinic dehydrogenase is strongly inhibited by some organic acids such as malonic acid which acts as a competitive inhibitor (1928). Their study has been extended by other workers and it was found, for instance, that oxalacetic acid even in a minute concentration (10^{-4} M) acts as a powerful inhibitor of this enzyme (Das 1937). The inhibitory effect of pyrophosphate was discovered by Dixon and Elliott (1929) and more recently studied by Leloir and Dixon (1937) who assigned it to the category of competitive inhibitors. Finally, it was shown recently by Hopkins and Morgan (1938) that succinic dehydrogenase is poisoned also by incubation with —S—S— compounds such as oxidized glutathione. They ascribe this inhibition to the oxidation of —SH groups of the enzyme the integrity of which seems to be essential for the normal catalytic activity of the enzyme. These results have been confirmed and extended by Hopkins, Morgan and Lutwak-Mann (1938).

On repeating some of these experiments we have found that all the substances such as metals, fluoride, malonate and pyrophosphate which inhibit the activity of succinic dehydrogenase inhibit also the reduction of cytochrome in presence of succinic acid.

To the above-mentioned inhibitors of the succinic dehydrogenase system of muscle we are able to add several other inhibitors which deserve special consideration, and which we propose to examine separately.

(2) *Effect of tetraethyldithiocarbamyldisulphide* (T.D.D.) *and analogous compounds*

The study of these inhibitors originated from experiments dealing with the effect of sodium diethyldithiocarbamate (D.D.C.) $(C_2H_5)_2NCSSNa$ on the aerobic oxidation of succinic acid by the muscle preparation. This reagent which is used for the detection and estimation of copper is a strong inhibitor of polyphenol oxidase.

The addition of this compound in very small concentration such as 3×10^{-4} M almost completely abolishes the aerobic oxidation of succinic acid by the complete system in presence of an excess of cytochrome *c* (figure 5).

The fact that this reagent has no effect on the cytochrome system suggests at first thought the possibility that it may combine with the dehydrogenase thus revealing the presence of catalytically active copper. This suggestion was, however, completely disproved by further study of the mechanism of this inhibition. The mere examination of the curves in figure 5 shows that the inhibition does not set in at once but develops during the first 10 min. and then becomes complete. Moreover, the facts that the inhibition takes place only in presence of an excess of cytochrome *c* and that the reagent has the property of reducing this component suggest that D.D.C. undergoes oxidation to a compound which acts as an inhibitor of the succinic dehydrogenase. This suggestion was amply confirmed by further experiments. We have found that heart muscle preparation in presence of cytochrome *c* rapidly oxidizes the reagent as shown in figure 6 to a product which strongly inhibits succinic dehydrogenase. The oxidation product thus formed is tetraethyldithiocarbamyldisulphide (T.D.D.). $(C_2H_5)_2N—CSS—SSC—N(C_2H_5)_2$ which can be obtained directly from sodium diethyldithiocarbamate by oxidation with iodine (Grodzki 1881; v. Braun 1902). The inhibition with D.D.C. is more marked if the enzyme preparation and cytochrome *c* are shaken with the reagent for 10 to 20 min. before the addition of succinic acid. This is clearly demonstrated by experiments (figure 7) showing that $4 \cdot 3 \times 10^{-6}$ and $1 \cdot 9 \times 10^{-5}$ M of this reagent inhibit under these conditions the activity of succinic dehydrogenase by 70 and 100% respectively.

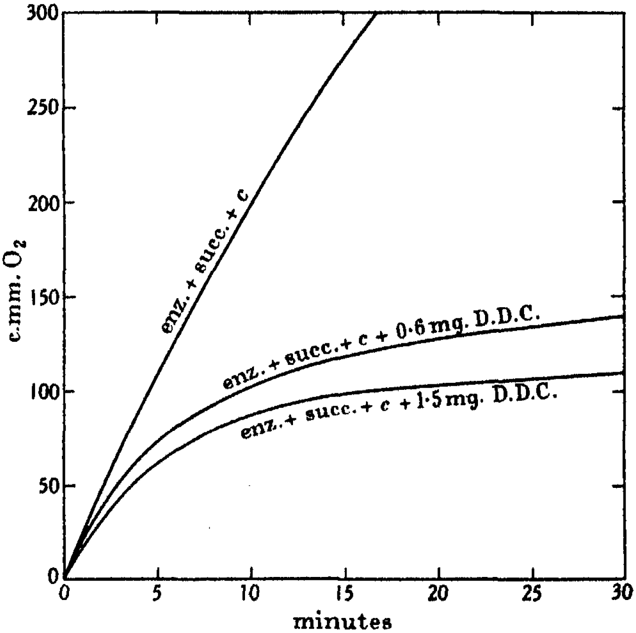


FIGURE 5. Effect of sodium diethyldithiocarbamate (D.D.C.) on succinic system.

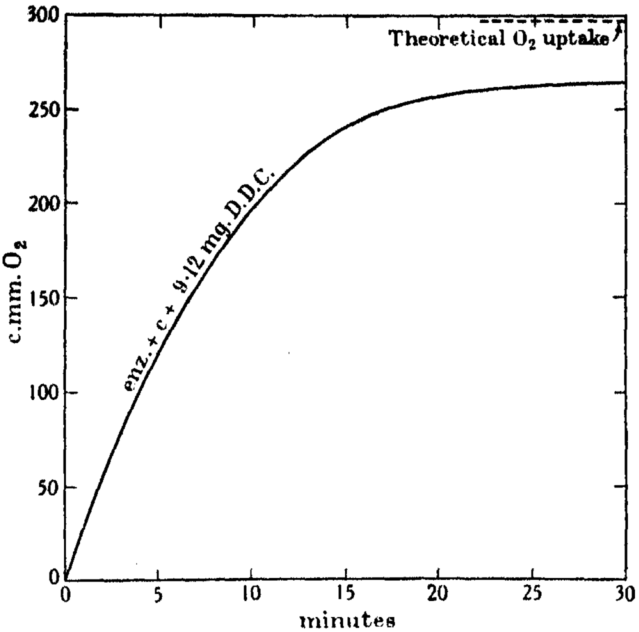


FIGURE 6. Oxidation of sodium diethyldithiocarbamate (D.D.C.) by the oxidase-cytochrome system to tetraethyldithiocarbamyldisulphide (T.D.D.).

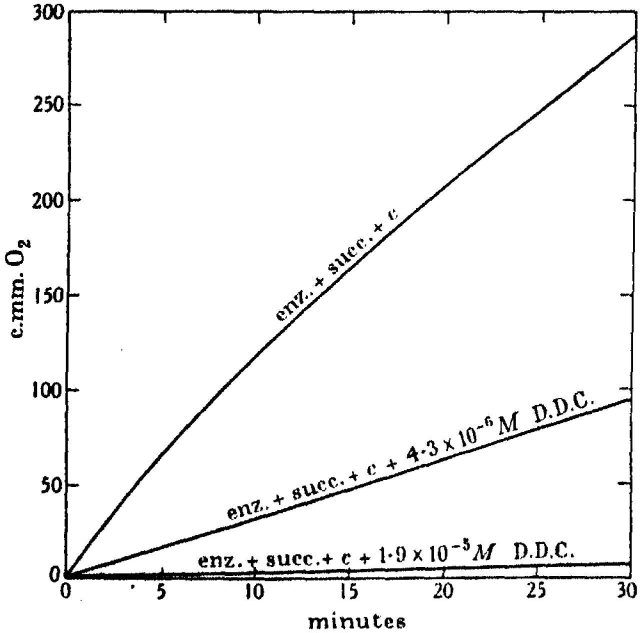


FIGURE 7. Inhibition of succinic system after 20 min. incubation with sodium diethyldithiocarbamate (D.D.C.).

The same degree of inhibition is obtained even if methylene blue is added to the oxidizing system. On the other hand, the oxidation of *p*-phenylenediamine in presence of this reagent is if anything slightly accelerated.

These results have been confirmed by using instead of the dithiocarbamate its oxidation product in a comparatively pure state.* While a 10^{-4} M solution of this substance (T.D.D.) produces an almost complete inhibition of the dehydrogenase without previous incubation, a concentration of 10^{-5} M under the same conditions has no immediate effect although after 20 min. incubation with the enzyme in the absence of substrate the activity is inhibited by 95 %. This experiment clearly demonstrates that very small concentrations of T.D.D. inhibit the enzyme only after a certain period of incubation. The inhibitory effect so produced is almost irreversible. The enzyme does not recover its activity even after a thorough washing with and without reprecipitation. The activity is not recovered after treatment with H_2S, KCN or $Na_2S_2O_4$. Only 2 hr. incubation of inhibited enzyme preparation with 0·1–0·5 M glutathione produces partial recovery of its activity. On the other hand 0·05 M glutathione added before the inhibitor completely protects the enzyme from inhibition by small concentrations of T.D.D. Similar and even more marked protection against this inhibition can be obtained by the addition of a small amount of boiled heart muscle preparation.

It is important to note that the alcohol treated enzyme preparations which have lost the property of reacting with cytochrome *c* and can still catalyse the oxidation of succinate by methylene blue, are hardly affected by this inhibitor. Thus such a preparation of $Q_{O_2} = 100$ has after 15 min. incubation at 39° C with 0·2 mg. of T.D.D. a $Q_{O_2} = 91$. Moreover, the addition of alcohol treated to untreated preparation protects the latter to a very great extent from inhibition (figure 8).

Inhibitory effects very similar to the above have been obtained with several other substances such as: (*a*) the oxidation product of potassium ethyl xanthate, (*b*) sodium tetrathionate and (*c*) potassium persulphate.

(*a*) Potassium ethyl xanthate, like diethyldithiocarbamate, undergoes oxidation in presence of cytochrome oxidase and cytochrome *c*. In a concentration of 10^{-3} M, after being shaken 20 min. with the enzyme preparation and *c* in absence of succinate, it produced an almost complete inhibition of succinic dehydrogenase.

A pure sample of the oxidation product (EtO.CSS.SSC.OEt) was prepared in the following way:

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* A sample of this compound in a crystalline state was kindly given to us by Dr B. C. Saunders.

A 10 % aqueous solution of potassium ethyl xanthate is treated with a saturated alcoholic solution of iodine added in slight excess. The oil so formed solidifies at 0°, is washed with water and rapidly pressed between filter papers. A saturated solution of this product in 50 % alcohol at 25° C is kept at 0° C for one week. The oxidation product diethoxydithiocarbonyldisulphide (EtO.CSS.SSC.OEt), which gradually crystallizes out as slender colourless needles, is rapidly washed with cold 50 % alcohol and dried in a desiccator; m.p. 30° C.

This compound was found to have a very strong inhibitory effect on succinic dehydrogenase both in presence of cytochrome *c* and of methylene blue. Thus 10^{-3} M of this substance completely abolishes the activity of the dehydrogenase while it does not affect the cytochrome system.

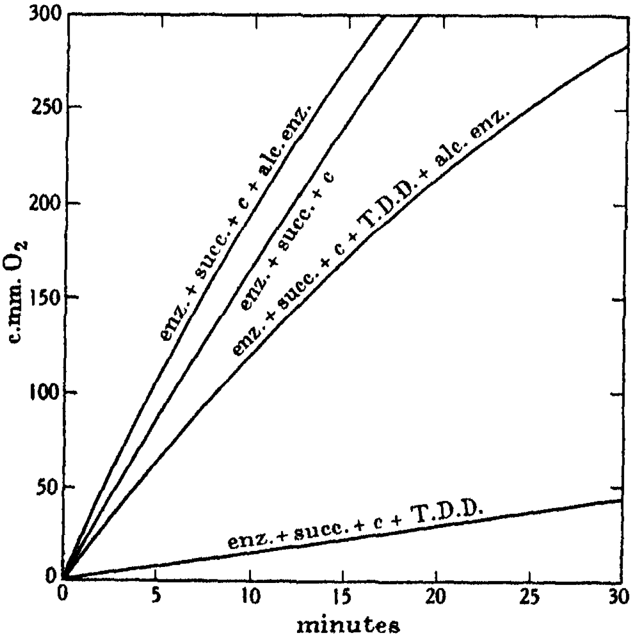

FIGURE 8. Inhibition of succinic system by 0·2 mg. tetraethyldithiocarbamyldisulphide (T.D.D.) and reduction of this inhibition by an alcohol treated enzyme preparation (alc. enz.).

(*b*) Sodium tetrathionate ($NaO—SO_2.S—S.SO_2—ONa$) the oxidation product of sodium thiosulphate in a concentration of 10^{-4} M inhibits the activity of succinic dehydrogenase by 90 %. Here again, the inhibition takes place only after 10–20 min. incubation of the enzyme with the inhibitor in absence of substrate.

(*c*) Potassium persulphate ($KO—SO_2.O—O.SO_2—OK$) in a concentration of 10^{-4} M inhibits almost completely the activity of succinic dehydrogenase. It has a strong inhibitory action even in 10^{-5} M solution.

The inhibition of succinic dehydrogenase by these substances is probably very similar to that produced by oxidized glutathione (GSSG) as was recently shown by Hopkins and Morgan (1938). Their results have been confirmed by Hopkins, Morgan and Lutwak-Mann (1938) for the same enzyme and by Rapkine (1938) for the enzyme catalysing the oxido-reduction between triose phosphate and pyruvate. According to these workers the mechanism of this inhibition consists in the oxidation by GSSG of —SH groups which are present in the enzyme and which are essential for its normal activity. This interpretation is strongly supported by their other findings, namely, (1) that the enzyme inhibited in this way recovers its activity on incubation with an excess of GSH or of cysteine, (2) that the enzyme can be inactivated also by other substances which react with SH groups such as alloxan, maleic and iodoacetic acids and (3) that malonic, succinic and fumaric acids protect succinic dehydrogenase from the inhibitory effect of GSSG.

The inhibition of succinic dehydrogenase by the substances examined in this chapter is probably of the same nature and may therefore be also explained by their ability to oxidize the —SH groups of the enzyme. On the other hand, these substances have another property in common, namely that their general molecular configuration is analogous to that of pyrophosphate which is considered to act as a competitive inhibitor by virtue of a molecular configuration which permits it to occupy the same active groupings in the dehydrogenase as succinic acid (Leloir and Dixon 1937). The very low concentration at which these substances exert a strong inhibitory effect indicates that their peculiar structure makes them easily accessible to the same groups of the enzyme which react with the substrate.

It is also possible that the inhibition by these substances is favoured by the very low concentration in our preparations of denatured proteins with unmasked —SH groups. In fact alcohol treated preparations rich in denatured proteins are not only unaffected by these inhibitors but protect the untreated preparations from the usual inhibition (figure 8).

The fact that the integrity of —SH groups seems to be an essential condition for the normal activity of succinic dehydrogenase indicates that these groups do not undergo reversible oxidation during the catalytic oxidation of succinic acid. In other words, the —S—S— groups, when formed, cannot act as hydrogen acceptors or carriers in the dehydrogenating mechanism of the substrate molecules. It therefore appears that —SH groups of this enzyme may only act as links between the enzyme and the substrate molecules and not as prosthetic groups taking part in the hydrogen transfer from the substrate to other components of the succinic dehydrogenase-cytochrome system.

(3) *Effect of coenzyme I*

The diphosphopyridine nucleotide in small concentrations ($0{\cdot}5 \times 10^{-5}$–$2{\cdot}5 \times 10^{-5}$ M) strongly inhibits the oxidation of succinate by the heart muscle preparation,* while it has no effect on the oxidase-cytochrome system. At first sight it appears as if coenzyme inactivates the succinic dehydrogenase by combining with some of its active groups. Further study of this inhibition did not, however, confirm this view but revealed an entirely different mechanism of this phenomenon which can be summarized as follows. The fumaric acid formed as the product of oxidation of succinic acid gives rise under the action of fumarase to malic acid which in presence of coenzyme added to the preparation undergoes a partial oxidation to oxalacetic acid. The latter, not being reduced or decarboxylated, acts as a very strong competitive inhibitor of succinic dehydrogenase (cf. p. 295). This interpretation is supported by the following experimental results:

(*a*) The inhibition as shown in figure 9 does not take place at once but develops gradually during the experiment. This lag period is required for the formation of a small amount of oxalacetic acid from the malic acid.

(*b*) Long preliminary incubation of the enzyme preparation with coenzyme in the absence of substrate does not shorten this lag period.

(*c*) Addition of small amounts of fumaric or malic acids reduces or abolishes completely this lag period (figure 10).

(*d*) In this respect, while *l*-malic shows a clear effect, *d*-malic acid, the oxidation of which is not catalysed by malic dehydrogenase, hardly affects the inhibition of the succinic system by coenzyme.

(*e*) Heart muscle preparations in which the activity of fumarase is abolished are no longer sensitive to coenzyme.

(*f*) Enzyme preparations obtained from tissues which, like brain, are capable of decarboxylating oxalacetic acid are not affected by coenzyme. Such preparations also protect heart tissue from the inhibitory effect of coenzyme.

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* The inhibition of the succinic system by cozymase was previously noticed by Potter (1939). A distinct effect was obtained by him only with very high concentrations of cozymase varying from 400 to 1600 μg. per manometric experiment. He has failed, however, to explain the mechanism of this inhibition and mentions that 'it cannot be stated at present which component of this system is inhibited by cozymase'.

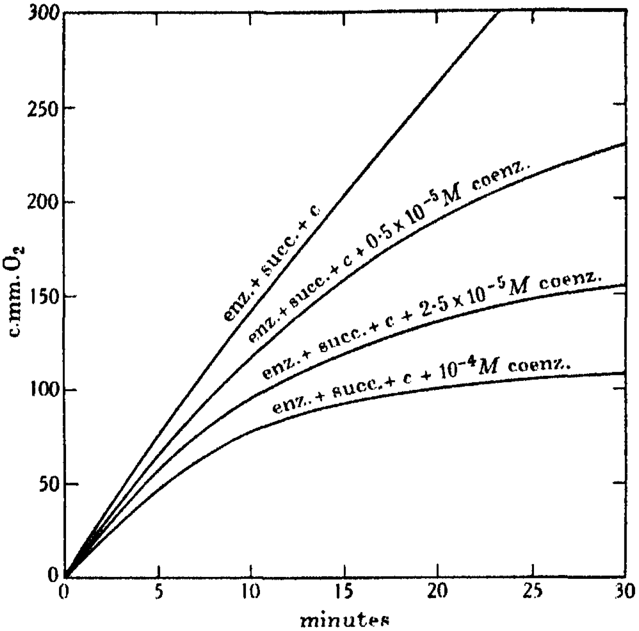

FIGURE 9. Effect of coenzyme I (coenz.) on succinic system.

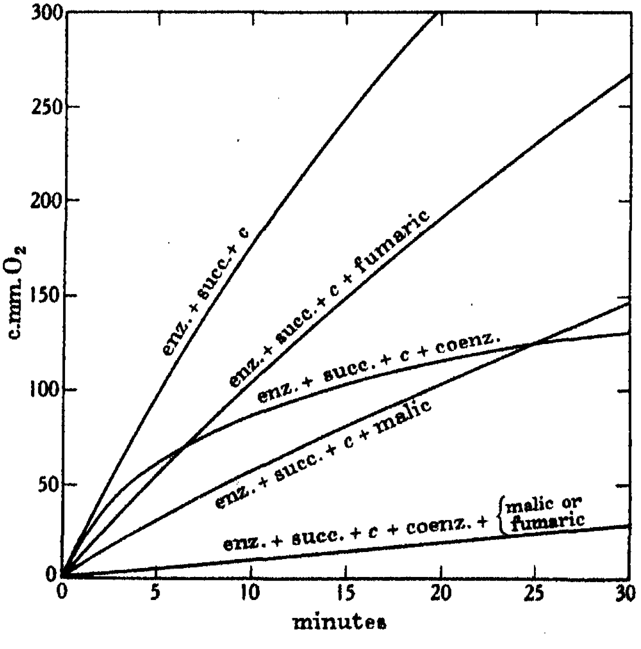

FIGURE 10. Effect of fumaric (0·01 M) and *dl*-malic (0·02 M) acids on the inhibition of succinic system by coenzyme I (10^{-4} M).

(*g*) Muscle preparation is also protected from this inhibition by glutaminic acid which combines with oxalacetic acid (figure 11).

(*h*) Coenzyme 2 which does not promote the oxidation of malic acid has a much less marked effect than coenzyme I.

These points clearly show that the inhibitory effect of coenzyme is not due to its reaction with any one of the components of the succinic system but to the formation of oxalacetic acid which is promoted by coenzyme and which acts as a strong competitive inhibitor of succinic dehydrogenase.

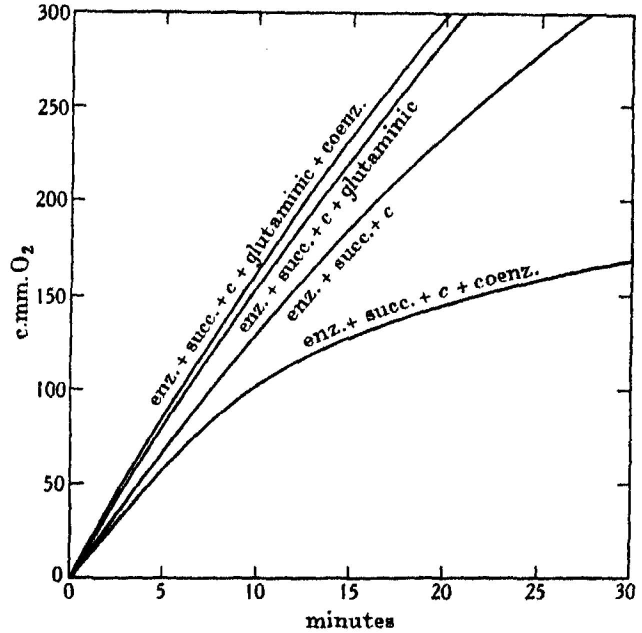

FIGURE 11. Effect of *dl*-glutaminic acid (0·02 M) on the coenzyme ($2 \cdot 5 \times 10^{-5}$ M) inhibition of succinic system of heart muscle preparation.

(4) *Effect of pyocyanine*

The oxidation of succinic acid is strongly inhibited by pyocyanine (figure 12) but this inhibition shows the following peculiarities.

(1) Pyocyanine in low concentrations, which strongly inhibits the activity of the complete succinic cytochrome system, has no effect on the oxidation of succinic acid through methylene blue.

(2) The addition of cyanide (10^{-1} M) to the system inhibited by a higher concentration ($1 \cdot 5 \times 10^{-3}$ M) of pyocyanine partly abolishes this inhibition and brings the activity of the system to the level of methylene blue.

Pyocyanine in presence of KCN acts, therefore, as an autoxidizable hydrogen acceptor similar to M.B. (figure 12). At lower concentrations (10^{-4} M) its action disappears.

(3) Glutaminic acid does not affect the inhibitory action of pyocyanine.

(4) Brain preparations treated with pyocyanine behave in every respect like muscle preparations.

(5) Incubation of tissue preparations with pyocyanine does not increase its inhibitory effect.

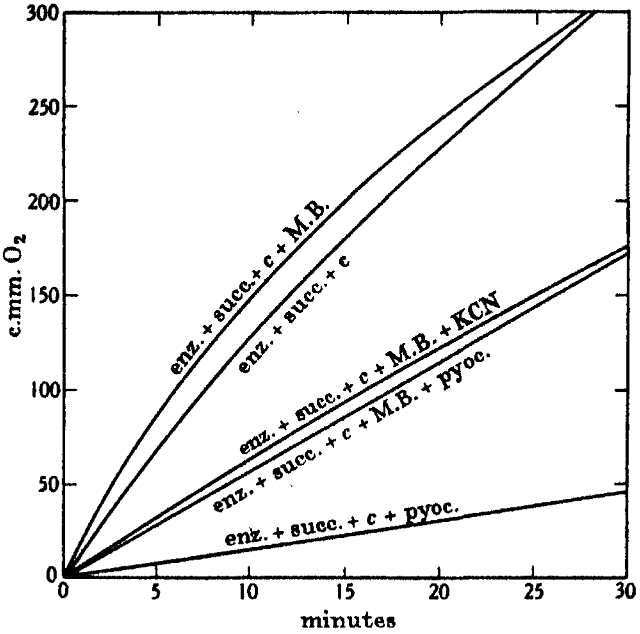

FIGURE 12. Effect of methylene blue on the pyocyanine ($1{\cdot}5 \times 10^{-3}$ M) inhibition of the succinic system; KCN – 0·1 M.

These results show that the inhibitory effect of pyocyanine, unlike that of coenzyme, is not due to the formation of oxalacetic acid but to a direct action of pyocyanine on succinic dehydrogenase. The inhibition by pyocyanine is probably due to its ketonic character. The fact that it functions in presence of KCN is in accord with the known lack of reactivity of its carbonyl group.

XII. SUMMARY AND CONCLUSIONS

1. The aerobic oxidation of succinic acid by tissue or by cell-free colloidal tissue preparations requires the co-operation of succinic dehydrogenase with the complete cytochrome system.

2. The catalytic activity expressed as the turnover number of cytochrome *c* in this system reaches a value of about 1420.

3. The non-autoxidizability of cytochrome components is a property intimately connected with their catalytic activity. In fact, when cytochrome *c* becomes autoxidizable it loses completely its catalytic activity both in the system catalysing the oxidation of succinic acid as well as in the system oxidizing *p*-phenylenediamine.

4. In tissue preparations oxidizing succinic acid the cytochrome system can be replaced by methylene blue the efficiency of which is, however, much lower than that of cytochrome.

5. Every preparation, which in presence of succinic acid reacts with cytochrome *c*, will also react with methylene blue. The reverse, however, is not true and preparations can be obtained which reduce methylene blue but not cytochrome *c*.

6. In some of these preparations both portions of the system: cytochrome-oxidase and succinic dehydrogenase are active independently and yet do not interact to form the complete system catalysing the aerobic oxidation of succinic acid.

7. These results suggest that either (*a*) the succinic dehydrogenase system reduces cytochrome *c* not directly but through an intermediary labile link such as cytochrome *b* or a hitherto unrecognized component of the system, or (*b*) the succinic system in such preparations has lost its accessibility to cytochrome *c* while it remains still accessible to the smaller and more diffusible molecules of methylene blue.

8. The aerobic oxidation of succinic acid by tissue preparations in presence of *c* is strongly inhibited by sodium diethyldithiocarbamate. This inhibition was found, however, to be caused not directly by this compound but by the tetraethyldithiocarbamyldisulphide $(C_2H_5)_2NCS.SS.SCN(C_2H_5)_2$ (T.D.D.) derived from the former by an oxidation catalysed by the oxidase-cytochrome system. T.D.D. in concentrations of 5×10^{-6} and 2×10^{-5} M inhibits the activity of the succinic system by 70 and 100% respectively. Similar inhibitions are obtained with tetrathionate, persulphate and the oxidation product of potassium ethyl xanthate.

9. The inhibitory effect produced by these substances is probably analogous to that of oxidized glutathione and due to the oxidation of

—SH groups of the enzyme the integrity of which seems to be essential for the normal activity of the enzyme (Hopkins, Morgan and Lutwak-Mann 1938).

10. The —SH groups of succinic dehydrogenase cannot therefore be considered as its prosthetic groups taking part in the hydrogen transfer from the substrate to other components of the system. They may act, however, as links uniting the substrate with the enzyme.

11. Coenzyme I in a very low concentration ($0{\cdot}5\times10^{-5}$–$2{\cdot}5\times10^{-5}$ M) strongly inhibits the oxidation of succinic acid. This inhibition is due to the formation, in presence of coenzyme, of a small amount of oxalacetic acid which acts as a very strong competitive inhibitor of succinic dehydrogenase.

12. Pyocyanine in a low concentration (10^{-3} M) strongly inhibits the activity of the succinic system.

13. The succinic dehydrogenase-cytochrome system even in the cell-free colloidal preparations behaves as a true respiratory system of the cell, showing a high catalytic activity and being affected by all inhibitors in the same way and to the same degree as the normal respiration of intact cells. The efficiency of the system depends, however, not only on the integrity of its components but also on that of the colloidal structure which supports them and assures their mutual accessibility.

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X-ray studies of the molecular structure of myosin

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[Plates 15–17]

The X-ray and elastic properties of myosin are found to resemble most closely, not those of natural keratin, but those of keratin that has suffered breakdown among the cross-linkages (including disulphide bridges) of the polypeptide grid. The supercontraction of myosin cannot be explained as due simply to disorientation of long thin units: it must involve a further folding of the polypeptide chain system. The fact that myosin and keratin are similar in both molecular configuration and elastic properties is discussed in the light of recent X-ray and chemical findings, and the X-ray interpretation is given of the denaturation of myosin.

INTRODUCTORY

X-ray studies of the molecular structure and properties of animal hairs (Astbury and Street 1931; Astbury and Woods 1933; Astbury and Sisson 1935; Woods 1938)* lead naturally to comparable studies of muscle tissue, if only because both types of structure are protein and fibrous, but particularly because both show similar long-range elastic phenomena and give similar X-ray photographs (Astbury 1933*a*, 1934). Previous X-ray studies of muscle (Herzog and Jancke 1921, 1926; Boehm and Schotzky 1930; Clark and Corrigan 1931; Boehm 1931, 1933; Worschitz 1934; Meyer and Picken 1937) do not appear to have brought out or made any use at all of this resemblance, the starting-point of the present investigations, though the closeness between the X-ray diffraction pattern of normal hair and that of relaxed muscle, washed and dried, is such as to imply immediately that the two structures depend for their properties on proteins of like molecular configuration.

The common photograph of animal hair,† horn, nails, spines, etc., arises from the insoluble fibrous protein *keratin*, in the form of long thin, submicroscopic 'crystallites' lying approximately parallel to the natural fibre axis. The crystallites are apparently of the nature of polypeptide chain-bundles, but the inference is that the chains are not normally in the fully extended configuration characteristic of *fibroin* of natural silk (Meyer and Mark 1928, 1930), but are in some regularly folded configuration that can be pulled out straight by stretching the fibre (Astbury 1930, 1931; Astbury and Woods 1930, I, II), since it is only then that a diffraction pattern is obtained analogous to that of silk: normally the pattern is quite different. In the presence of water at ordinary temperatures the fibre is reversibly elastic over a great range, and the change from one type of X-ray photograph to the other and back again always accompanies stretching and recovery. There is presumably a reversible intramolecular transformation between a shorter protein molecule (α-keratin) and an isomer (β-keratin) of probably roughly twice the length.

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* It will be convenient to refer to these papers in the text simply by the numbers I, II, III and IV, respectively.

† In what follows the word "hair" means any mammalian hair.

The remarkable resemblance between the X-ray photographs of unstretched hair and relaxed muscle, washed and dried, shows at once not only that the chief protein constituent of muscle too lies in the form of long thin, submicroscopic chain-bundles approximately parallel to the natural fibre axis, but also that the protein chains are not normally in the fully extended configuration, as perhaps might be expected from the contractile properties of the structure, but are in the folded α-configuration. A few years ago such a conclusion might have sounded paradoxical, because hair is one of the most beautifully extensible structures known and muscle is nothing if not contractile—or at least it might have been used as an argument against the idea that muscle contraction is due to a contraction of the protein molecules themselves (Meyer 1929, 1930); but the paradox has been resolved by the discovery (II) that hair also has long-range contractile properties, latent in the ordinary way, but functioning when certain linkages between the side-chains of neighbouring main-chains are broken down. Keratin is thus not only elastically extensible; its normal equilibrium form is really a metastable phase whose inherent powers of contraction are inhibited by its cross-linkages. If the latter are disrupted, as shown in the X-ray photographs, the system shortens when its temperature is raised in the presence of water—or even in the cold under the action of dilute alkalis—and is now reversibly elastic over a greater range still. To distinguish this phenomenon from the ordinary elastic recovery of stretched hair, it has been proposed to call it 'supercontraction' (II). The supercontracting form of keratin, then, can either be stretched by about 100 % or contracted (in steam) by about 30 %.

We may fairly conclude, therefore, that there is neither paradox nor accident in the correspondence between the X-ray photographs of muscle and hair—the chance of such close accidental agreement seems in any case remote—but that underlying it there is some kind of genuine *molecular* correspondence. The characteristic protein of each appears to exist normally in the folded α-configuration, both have long-range contractile powers (the supercontraction of hair being the counterpart of the contraction of muscle), and both—a critical deduction—should be capable of elongation into similar β-modifications. When first this deduction was made the writers tried without success to change the muscle substance by stretching (1933), but now the transformation has been accomplished (Astbury and Dickinson 1935*b*), as will be described in the next paper of this series; and prior to this it had been brought about without difficulty in the isolated muscle protein (Astbury and Dickinson 1935*a*).

The chief muscle protein *myosin* comprises about 70% of the total soluble protein (Weber 1933, 1939; Bate Smith 1937), and it is present as a swollen solid, or gel, in a medium which probably contains all the other soluble proteins (globulin X, myogen and myo-albumin) in solution (Bate Smith 1937). It may be extracted with KCl solution, for instance, and re-precipitated from the resulting sol in a pure, undenatured state (Edsall 1930). Boehm and Weber (1932) prepared myosin threads in this way and were the first to show that such threads, when oriented by stretching, give an X-ray photograph like that of muscle itself. They did not observe however, and make no reference to the possibility of, an α-β transformation of the kind discovered in keratin, though Weber (1933, 1934) made a valuable study of the optical and elastic properties and demonstrated that at a certain stage of extension there must begin an elastic change in the actual myosin molecules. A detailed X-ray study of myosin follows below, and it will suffice at this point to say that there is no doubt now that the main component of the X-ray photograph of muscle arises from elongated units of myosin, oriented, like keratin in hair, practically parallel to the direction of the natural fibres. There are present haloes, of course, from the fluid contents in the case of undried muscle, and also generally weak reflexions from the connective tissue; but to all intents and purposes the X-ray photograph of muscle, whether living or dead, is a myosin photograph.

The optical properties of muscle (v. Ebner 1882; Hürthle 1909; Schmidt 1924, 1937; Stuebel 1923, 1928; v. Muralt 1932, 1935; Weber 1933, 1934, 1939; Noll 1934; Langelaan 1934, 1936; v. Studnitz 1935; Buchthal 1936, 1937; Fischer 1936) are also chiefly those of its myosin constituent, as in fact are so many of its properties, and it is nowadays generally agreed that this protein is the central working substance and the real seat of its elasticity. The logical procedure then, from the present point of view, is to commence with a detailed examination of free myosin and afterwards continue with the X-ray study of muscle proper. This first paper is thus devoted to myosin, and the next paper will deal with muscle itself.

MATERIAL

The first experiments (1935*a*) were performed with films made from myosin sols provided by Dr E. C. Bate Smith (1934) of the Low Temperature Research Station, Cambridge, but subsequently regular supplies were made by Dr K. Bailey (1937*b*), who prepared rabbit myosin from the hind limb muscles and longissimus dorsi, lobster myosin from the abdominal and pincer muscles, and *Mytilus edulis* myosin from the foot retractor muscle.

After various trials (chiefly to eliminate incipient spontaneous transformation into the β-form—see below), the best procedure for making myosin films suitable for X-ray and elasticity experiments was found to be as follows: The Edsall buffer extract was precipitated in 20 vol. of water at 0° C, centrifuged off and washed once with water. To the washed gel was added solid KCl to 8–10 % concentration, and the sol thus produced was filtered through paper pulp, precipitated again, centrifuged and washed, taken up with KCl as before, and again filtered. As a rule the protein concentration was about $1\frac{1}{2}$ %. The sol was then spread thinly on a clean glass plate and dried on a warm plate in a current of air such that the surface temperature was 23° C. Each film took some 15–20 min. to dry, and the KCl was not washed out of each separately, but about three thicknesses were built up before washing in distilled water and drying again before another addition of sol. In this way sheets of various final thicknesses were built up and stored, still adhering to their glass plates, till needed. Success seems to depend on making each constituent film *thin*, so that it dries as a whole without unduly retarding evaporation from the interior. The *Mytilus* myosin films were prepared in a similar manner, except that the sol concentration was 0·6 %.

On moistening a film on its glass plate, strips one or two millimetres wide could be cut by means of a scalpel and ruler, and then lifted off with the aid of the scalpel blade.

THE α- AND β-PHOTOGRAPHS AND THE INTRAMOLECULAR TRANSFORMATION

Myosin film prepared as just described and photographed with the X-ray beam perpendicular to the surface gives a ring photograph (figure 1, plate 15) closely resembling that of disoriented α-keratin; but with the beam parallel to the surface it gives a pseudo fibre photograph, intermediate between figures 1 and 2, plate 15, indicating that the structure is one of elongated units lying roughly parallel to, but randomly disposed in, the plane of the film (see below). On moistening and stretching, the first thing that happens is that the units are pulled parallel to the direction of stretching, so that an *oriented* α-photograph gradually develops even with the X-ray beam perpendicular to the surface, and especially when it is parallel to the surface. The process of orientation approaches completion when the myosin strip is about 1½ times its initial length, but it is then complicated by the onset of the intramolecular transformation into β-myosin; which means that it is not possible to get a well oriented, pure α-photograph by this method.

The difficulty was overcome by the discovery that after swelling the strips for a few minutes in 1 % NaOH solution, the internal cohesion is so much reduced that they can be stretched to three or four times their original length without breaking or appreciable transformation, and on washing thoroughly and drying at the stretched length, a very good oriented α-photograph is obtained (figure 2, plate 15). Swelling in N/10 HCl works similarly, but the α-photograph is not quite so good and shows traces of the β-form.

Figure 3, plate 15, is an exact comparison between the X-ray photographs of oriented α-myosin (obtained as just described) and oriented α-keratin (horn, photographed parallel to the surface, I, II). The two specimens were similar in shape and size and were exactly interchangeable on the spectrometer, and the comparison was carried out on one and the same photographic film according to the technique described in II. The two quadrants of the myosin photograph are marked *M*, and those of the horn photograph *H*, and it will be seen that there is a very close correspondence indeed. If anything, the side-chain reflexion of myosin (on the equator) is slightly nearer the centre and appears to correspond to a spacing of about 9·9 A. The spacing of the meridian reflexion is close to 5·1 A.

Uniform strips of untreated film usually stretch in water or water vapour to about three times their initial length without breaking, but the transformation into β-myosin is never complete owing to lack of internal cohesion, though it generally progresses farther on stretching in water vapour, as would be expected. The maximum extension (about 200 %) corresponds approximately with that given by fully oriented keratin (about 100 %), because the act of orienting the units lying disoriented in the plane of the film first involves an elongation of 57 % (I), and then this new oriented length is roughly doubled by the intramolecular transformation. It is, however, hardly possible to obtain such uniform elasticity data from myosin strips as may be obtained from animal hairs, partly owing to lack of uniformity in the strips and partly on account of variations in the properties of the myosin itself, arising from our present incomplete knowledge of its structure and the best ways of dealing with it in different circumstances. In general, for instance, there seems to be inevitably a certain amount of internal (intermolecular) slipping when extracted myosin is moistened and stretched, and the elastic properties depend to some extent also on the age of the preparation. Again, the elastic recovery of myosin film prepared by evaporation on a flat surface is always incomplete by a rather vague amount because of the orientation that first takes place on stretching, though the residual extension, which may be anything from perhaps 30 to 70%, is of the right order (theoretically 57%). Recovery in water vapour is incomplete, even allowing for the orientation effect; but a few hours' soaking in water usually suffices to bring back a stretched strip to about the length to be expected on making this allowance.

The indications then from ordinary stretching and recovery experiments are that myosin in the presence of water is definitely reversibly elastic over a range of roughly twice its oriented length: observed irregularities are no more than may legitimately be referred to internal slipping, overlapping of the intramolecular transformation with orientation phenomena, and the elastic after-effect so particularly associated with structures built from chain-molecules.

As already mentioned, the transformation from the α- to the β-form of myosin, which is the molecular basis of its long-range elasticity, is never quite complete in the X-ray photographs when strips of film are stretched in water or water vapour, but practically complete reversal from the β- to the α-photograph is obtained on leaving the stretched protein to recover sufficiently long in water, even though it may have been kept dried for some time at its stretched length. It was thought originally (1935*a*) that this is not so, that drying stretched myosin partially destroys its power of reverting from the β- to the α-form; but later experiments have shown that though failures undoubtedly do occur, they are almost certainly to be ascribed to the shortcomings of the myosin preparations and their subsequent treatment.

An interesting effect was observed when myosin strips, stretched in water, were photographed in the wet state. At intermediate extensions there is practically no β-pattern to be seen, but only the water halo and a faint α-pattern with unusually vague side-chain reflexions. The β-pattern may be seen at higher extensions—and, of course, quite clearly always if the specimen is allowed to dry and then photographed again—but it is evident that the penetration of water between the main-chains, as shown by the lack of definition and the spreading of the side-chain reflexions for instance (Astbury and Lomax 1935; II), emphasises the tendency of the β-form to return to α and interferes with regular diffraction. The effect is presumably similar to what occurs on raising the temperature of stretched rubber: the thermal vibrations in the latter case finally bring about such departures from crystalline regularity that the X-ray fibre pattern is destroyed. It is known also that strong inter-chain swelling leads to a similar result (cf. Astbury 1933*b*).

For the purpose of exact comparison with keratin it was necessary to obtain a more perfect β-photograph than is given by stretching disoriented film, and this object was attained by using strips already brought into the oriented α-form by stretching in dilute caustic soda, as described above. Such oriented strips may be stretched in water vapour generally to rather more than twice their initial length, and they contract again quite quickly and powerfully when afterwards released in water. If they have been stretched by no more than about 100 %, elastic recovery in water is perfect; but if they have been stretched beyond this there is some residual elongation, indicating that internal slipping has occurred (see below). X-ray photographs show a nearly complete transformation into β-myosin before breaking, and there is almost perfect return to the α-form on contraction —in fact these oriented strips of myosin are obviously reversibly elastic over a range of about 100 %, and apparently, like mammalian hairs, they may be made to undergo the α-β transformation as often as required.

The final β-photograph shown in figure 4 (plate 16) was obtained from oriented strips first stretched by about 70 % in water vapour and then immersed, while still held stretched, for ten seconds in water at 80° C. The action of hot water will be discussed more fully below: the effect here was to complete the intramolecular transformation by means of a sudden increase of internal tension at constant length, as in the isometric contraction of muscle. Figure 5 (plate 16) is a composite comparison photograph (cf. figure 3) of β-myosin so obtained and β-keratin (horn at 70 % extension). Again it is seen how closely the two structures correspond from the point of view of X-rays: the only noticeable spacing difference lies in the side-chain reflexions, that of β-myosin being slightly nearer the centre.

X-ray photographs of oriented strips of myosin stretched to the limit in water vapour show also a phenomenon previously discovered in keratin stretched in hot water or cold dilute alkali—the so-called 'smear lines', or 'spreading along the hyperbolae' (II), which consists in this case of a drawing-out of every spot except those belonging to the zone [001]. The zone-axis [001] corresponds to the side-chain direction, and the inference is that in both structures high extensions are accompanied by a disruptive disturbance in that direction, though if only water is used to facilitate extension, higher temperatures are needed to bring about the effect in keratin.

Mr H. J. Woods kindly took for us a number of load-extension curves on specially prepared myosin strips about 1 mm. wide and 0·15 mm. thick which had previously been stretched in 1 % caustic soda solution to an extension of 275 % and then washed for 24 hr. and dried at the stretched length. They were stretched in water vapour at a rate of extension of 20–25 % per min. and gave curves essentially similar in form to those given by keratin (II). Three examples, (1), (2) and (3), are shown in figure 6, together with the load-extension curve (4) of 'generalized' Cotswold wool in cold water; that is, wool stretched first in dilute caustic soda to about twice its initial length, allowed to 'supercontract', then washed free from alkali, and finally stretched and allowed to contract again in cold water till the limiting reversibly elastic state has been attained and the load-extension curve has moved over to the left as far as possible (II). In this state side-chain breakdown (corresponding to the 'spreading along the hyperbolae' in the X-ray photographs) has been brought to a maximum without seriously injuring the keratin 'grid', and the fibre may be stretched in cold water up to about twice its initial length and contracted again as often as desired, while the X-ray photographs show the intramolecular transformation accordingly.

No great significance may be attached to the absolute values of the elastic constants given by the myosin curves in figure 6, owing to imperfections in the strips and the undefined variations already mentioned; but it will be clear enough that the keratin and myosin curves are similar in type, the sudden rise at the end of the Hooke's Law region corresponding to the onset of the intramolecular transformation, the almost vertical part to the bulk of the α-chains being transformed into β as soon as the critical load is passed, and the gradual bending over to the right towards the end to the heterogeneity of the structure and the progressive taking up of the load by chains that have already been transformed (II). Curve (2) records a restretching of the strip that gave curve (1), after it had contracted in water to within about 4 % of its initial length, while curve (3) represents the fourth stretching of another strip that had previously recovered within 1 %.

The second upward rise of curves (1) and (2) after about 100 % extension almost certainly corresponds to internal slipping when the transformation has been completed, for the further one succeeds in stretching oriented myosin beyond this extension the less perfect is the recovery on contraction. This effect had not previously been detected in keratin, the fibres always breaking in steam at about twice their unstretched length (II); but now Woods has so improved the technique that he has observed a similar inflexion with various keratin fibres too.

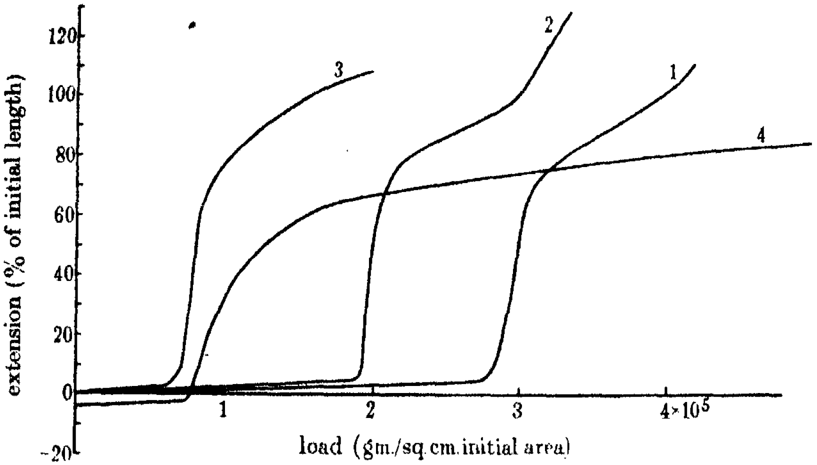

FIGURE 6. Load-extension curves (1), (2) and (3), of oriented myosin strips compared with that of 'generalized' Cotswold wool (4). (H. J. Woods.)

SELECTIVE ORIENTATION ON PRESSING

It has been described in III how moist keratin fibres may be stretched into the β-form simply by squeezing laterally, and how, *when the temperature is raised*, there follows also side-chain breakdown that permits the β-crystallites to rotate about their long axes and orient themselves with the side-chains perpendicular to the plane of flattening. The β-crystallites are tablet-shaped, but not in the sense that perhaps might be inferred from the fact that they are piles of flat polypeptide grids: the grids are relatively so narrow in the side-chain direction, so long in the main-chain direction, and so many are grouped together in the direction of the 'backbone spacing' (the picture is one of a high pile of strips of ribbon, each strip corresponding to a grid), that they lie *transversely* in the tablets; and once side-chain breakdown has taken place and so freed the interior of the effectively continuous complex to which the properties of the virgin keratin fibre conform, lateral pressure brings the side-chain spacing perpendicular, and the backbone spacing parallel, to the plane of flattening. (It was in this way (III) that it was first proved directly that the side-chain and backbone spacings are perpendicular to each other, as had already been inferred indirectly.)

Similar changes can be brought about in moist myosin by pressing *in the cold*. A convenient technique is to squeeze myosin film between two small pieces of thick plate glass held in Hofmann screw clips, and then to cut up the thinner film so produced and build it up into the form of a rectangular block, so that photographs may be taken in each of three directions at right angles. Squeezing alone causes disorientation in the plane of flattening, but this may be avoided by stretching and pressing alternately, so as first to produce the β-form more directly and then to preserve the orientation as much as possible. Figures 7–9 (plates 16, 17) were obtained in this way, the already oriented myosin strip being first stretched by 125 % in water vapour at room temperature. They should be compared with corresponding photographs of keratin pressed in steam (III, plate 11): the resemblance is very close indeed. In figure 7 (plate 16) the X-ray beam is parallel to the plane of flattening and perpendicular to the fibre axis, which brings out the side-chain reflexion strongly and leaves the backbone reflexion very weak. The photograph shows also clearly the 'spreading along the hyperbolae' that arises from a spacing disturbance in the side-chain direction only. In figure 8 (plate 17) the X-ray beam is perpendicular to both the plane of flattening and the fibre axis, which now brings out the backbone reflexion strongly and leaves the side-chain reflexion weak; while in figure 9 (plate 17) the beam is parallel to the fibre axis, which reveals directly the side-chain and backbone spacings standing at right angles to each other.

THE ACTION OF WATER AT HIGHER TEMPERATURES: SUPERCONTRACTION

It will be convenient to consider first hotter water or steam. In the X-ray photographs the effect observed is essentially one of sudden conversion into the β-form, and this is accompanied by contraction or development of tension in the specimen according to whether it is free or not. This is similar to what is observed in *stretched* keratin when treated with hot water or steam—or more generally, in keratin that has been brought into the labile (supercontracting) state by appropriate side-chain breakdown. Steam has no such action on normal, unstretched keratin, the X-ray photograph and fibre length remaining substantially unchanged.

The simplest application of the effect, long used in keratin studies (I, II), is enhancement of the β-form and improved orientation in the β-photograph when stretched hair is exposed to steam. This phenomenon arises from a combination of causes: the rapid transformation from α to β under the joint influence of stress and temperature, hydrolytic breakdown among the cross-linkages of the grid, and the tendency of the now labile grid even to supercontract. The result is a momentary development of tension which leaves an improved orientation of those parts of the structure that are better organized (see II). With myosin the internal stresses and changes set up by the action of steam, though analogous, are of a still more violent nature: even with stretched oriented strips there is some disorientation of the β-form that is suddenly produced. In fact, as has already been mentioned, it was found convenient to use water no hotter than about 80° C in order to improve the β-photograph obtained by stretching in water vapour at room temperature. If a piece of ordinary α-film is exposed to steam it contracts almost instantaneously by about 20% (linear) if free, and whether free or not gives then a β-photograph: it is not necessary to stretch the myosin first, as it is with keratin; it behaves naturally like the supercontracting form of keratin. It shows directly, too, another striking effect that can be brought about in keratin only by means of a special technique. The instantaneous internal tensions are apparently so strong that they pull over the tablet-shaped crystallites and bring them to lie with the side-chains roughly perpendicular to the plane of the film, just as though the film had been subjected to external pressure, as in the experiments described in the previous section. Rudall (1936) first obtained such a result with keratin by stretching various specimens in steam, not longitudinally but laterally. It was a somewhat surprising discovery, but it is reasonable enough on considering the matter; and knowing from the compression experiments that the myosin crystallites also are tablet-shaped, there seems no reason to doubt that sudden tensions developed in all directions in the plane of the film must tend to pull the crystallites flat.

Figure 10 (plate 17) illustrates the phenomenon in a piece of α-film exposed to steam for one second and then photographed with the X-ray beam parallel to the plane of the film. It will be seen that not only has the α-form been almost completely converted into β, but also that there is a definite concentration of the backbone reflexion on the meridian of the photograph, indicating that the corresponding spacing tends to lie parallel to the film, as though the latter had been pressed.

Later it was found possible to imitate Rudall's experiments even more closely, simply by stretching a wide strip of α-myosin film by about 125% in water vapour at room temperature. The stretched strip was then cut into other strips at right angles to the direction of stretching, built into a block, and photographed with the X-ray beam parallel both to the plane of the film and to the direction of stretching. The photograph so obtained was similar to figure 9, in that it showed the side-chain spacing tending to lie perpendicular, and the backbone spacing tending to lie parallel, to the plane of the film. Thus once again an effect that can be brought about in keratin only with the aid of hot water or steam was found to be realizable in myosin even at ordinary temperatures.

The well-known 'setting' effect of steam on stretched keratin (I, II) also finds its counterpart in myosin, for once a strip of β-myosin has been exposed to steam it loses completely its power of contracting again to the α-form; and that new cross-linkages are quickly set up, as they are more slowly when stretched hair is steamed, is shown for example by the fact that the disorientation mentioned above cannot be reduced appreciably by further stretching.

The maximum supercontraction so far attained with keratin fibres (II; Woods 1933) is of the order of 50 %; and assuming something similar for myosin fibrils lying parallel to the surface of a film, it is possible to estimate the order of contraction to be expected when such a film is exposed to steam. Consider a square of film of side a_1 and thickness t_1, and suppose it contracts to a square of side a_2 and thickness t_2. Then since the volume of myosin remains practically constant both on extension and supercontraction (this is true for keratin also, II), we have $a_1^2 t_1 = a_2^2 t_2$. But for a single chain-group or fibril, $t_1^2 l_1 = t_2^2 l_2$, where l_2/l_1 is the ratio of the supercontracted to the normal length: whence

$$a_2/a_1 = (l_2/l_1)^{\frac{1}{4}} = (0{\cdot}5)^{\frac{1}{4}} = 0{\cdot}84.$$

Thus arguing from keratin we should expect a supercontraction of at least 16 %, and actually about 20 % or a little more is usually observed with film prepared as described above. This corresponds to a undirectional supercontraction of about 60 % for myosin as compared with the 50 % of keratin, and it will be shown below that it is possible to realize supercontractions of this order with oriented strips of film.

The calculation assumes that the supercontraction does not involve disorientation of the chain-groups out of the plane of the film: such an effect undoubtedly does occur to some extent, and it may be that it accounts in part for the observed supercontraction being rather higher than the theoretically predictable value.

Completely disoriented myosin, of course, shows no supercontraction; and in this connexion it is worth mentioning an experiment devised to demonstrate that the selective orientation of myosin (and of gelatin, too, for example) found in films made by drying a pool of sol on glass is essentially an effect of drying under tension, and not one of attraction between the side-chains and the glass. There is enormous contraction on drying a pool of sol, but the area remains constant and so accommodation is reached by pulling the elongated chain-groups down as flat as possible—in fact, finding by X-rays such selective orientation in a dried film is one of the simplest proofs that the molecules or molecular groups from which it is formed are longer in one dimension than another. (Films of sodium thymonucleate (Astbury and Bell 1938) afford another impressive and more recent example of the principle.) A myosin film was made by drying a pool of sol on the surface of mercury, so as to allow greater freedom of contraction in all directions, and strips cut from it were tested as before for supercontraction in steam. Only about 9 % was obtained, instead of the usual 20 % or so, indicating that the chain-groups had been left, on drying, in a state approaching more to complete disorientation.

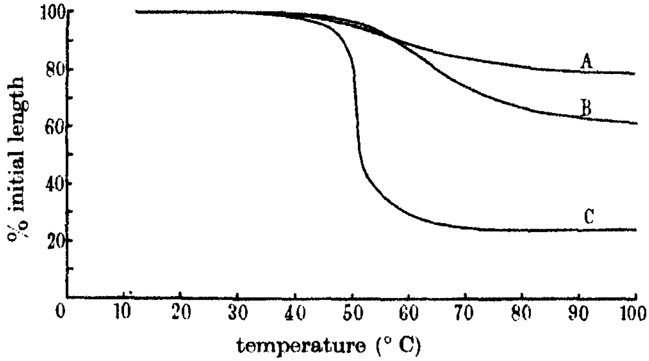

FIGURE 11. *A*, supercontraction of α-myosin film disoriented in the plane of the film; *B*, supercontraction of oriented myosin film, and thermal contraction of frog's sartorius muscle previously washed and dried; *C*, thermal contraction of foot retractor muscle of *Mytilus edulis* previously washed and dried.

Myosin film first commences to supercontract in water at about 40° C. Figure 11*A* is a typical curve illustrating the development of supercontraction with rising temperature in strips of film disoriented in the plane of the film. Strips about 3 cm. long and 1–2 mm. wide were removed from the glass plate with every care to avoid accidental stretching and were first washed in running water for 3 days. A complete experiment was carried out on one and the same strip, the procedure being to immerse it for a few minutes in hot water of known temperature, and then in water at room temperature, the length measurement being taken after the latter operation and always on the wet strip.

Myosin film oriented by stretching in caustic soda shows of course much greater supercontraction than film still disoriented in one plane: as much as 40 % may be realized in boiling water. Fig. 11*B* illustrates the development of the effect as the temperature rises above about 40° C. For this experiment disoriented strip was first stretched in 1 % caustic soda solution to about 2½ times its initial length, washed while still held stretched for 2 days in running water, then cut from the stretching frame and left free in distilled water at room temperature till ordinary contraction (some 10 %) had ceased. The successive immersions and length measurements were carried out as just described for disoriented strip.

X-ray and related studies of muscle proper will be discussed in the next paper of this series, but for comparison with the properties of extracted myosin it will be convenient to interpolate here some observations on the thermal contraction of washed and dried muscle. The myosin used for curves *A* and *B* of figure 11 was prepared from striated muscles of the rabbit (see above), and it is of obvious interest to compare curve *B* with the thermal contraction curve of washed and dried myosin *in situ* in a striated muscle. A frog's sartorius muscle, held at its body length, was washed for 2½ hr. in running water and allowed to dry over-night: next day it was re-wetted thoroughly and its thermal contraction measured by means of the technique used for the myosin strips. *It gave a series of points so closely conforming to curve B that the same curve suffices for both sets of observations.* Another sartorius muscle, washed immediately after removal and examined at once for thermal contraction without first drying, also gave points fitting closely on the same curve.

Figure 11*C* illustrates the thermal contraction of a washed and dried plain muscle, the foot retractor of *Mytilus edulis*. The shell of the mussel had been prised open* some time previously and its contents washed thoroughly in running water and allowed to dry *in situ*. The foot retractor muscle was cut out and soaked well in water before the actual thermal observations, for which it was given immersions of 1 min. each in hot water, except for a 3 min. immersion at 30° C. It will be seen that for this plain muscle contraction commences rather below 40° C, develops more rapidly with rising temperature, and finally amounts to as much as 75 % of the initial length—properties that we need not discuss for the moment.

When followed by X-rays, the increasing supercontraction of oriented myosin with rising temperature is found to proceed along lines very much as might be expected from X-ray studies of the supercontraction of keratin

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* Since the foot retractor muscle is almost parallel to the hinge of the shell, this operation stretches it very little; and in any case it was given an opportunity of contracting again in cold water before the thermal contraction tests.

(II), if account is taken of the greater sensitivity of myosin revealed by the various observations reported above. So long as steam or boiling water is avoided, or at least its use is cut down to a minimum, it is possible to supercontract keratin by perhaps 25 % or so without the β-form becoming obvious in the X-ray photographs, and even without marked disorientation of the α-pattern: disorientation of the α-pattern does set in gradually, it is true, but the degree of disorientation is always inadequate to explain the observed supercontraction in terms of disorientation alone. It lags behind so much that we can hardly escape the conclusion—especially in view of the X-ray and elastic properties of keratin over its normal range of extension and contraction—that supercontraction is simply the β-α contraction carried to a further stage by a continuation of the process of folding polypeptide chains. Owing to the ultimate inhomogeneity of the structure (variations from point to point in size, perfection, and maybe actual chemical constitution of the molecular aggregates), changes take place both in series and in parallel: molecular folding takes place in series as long as possible and will be confined at first to the less organized regions; but in time accommodation must be brought about by the gradual disorientation of less responsive regions in parallel.

The β-form, much disoriented, becomes definite in X-ray photographs of supercontracted myosin at a temperature as low as about 55° C, as opposed to about 90° C for keratin, and thereafter steadily becomes more prominent with rising temperature or longer treatment; but the α-form is still evident at 65° C, when the supercontraction amounts to about 20 %, and is still no more than perhaps half-way towards complete disorientation. Below supercontractions of about 10 % the disorientation of the α-form is insignificant.

Supercontracted myosin is reversibly elastic from its new shortened length, just as supercontracted keratin is, though there is the usual small amount of internal slipping at higher extensions (see above). As in keratin too, the higher ranges of extensibility are restricted to some extent when β-form is present, presumably owing to the formation of irregular cross-linkages.

Myosin supercontracts also in caustic soda solutions, in which respect it again resembles, not normal keratin, but keratin that has been subjected to side-chain breakdown by first stretching in hot water or cold dilute alkali: in both cases, by avoiding higher temperatures, supercontraction may be brought about without producing any β-form at all, and the disorientation of the α-pattern is again inadequate to explain the effect quantitatively.

For the experiments on supercontracting myosin in dilute caustic soda strips of film disoriented in one plane were first stretched in 1 % caustic soda to about 2½ times their initial length, washed for a day at the stretched length, dried, cut free, and finally allowed to contract as much as possible in the normal way by leaving for several days in water. After redrying they were cut into pieces of known length, rewetted and remeasured wet, and then immersed for 5 min. each in caustic soda solutions of various strengths, after which they were washed for several days in water, again measured wet, allowed to dry as freely as possible, and finally measured again dry. Table 1 shows a typical set of results, for which, however, no high accuracy is claimed, particularly on account of the difficulty of drying myosin freely without distortion. It will be seen that the maximum supercontraction obtainable in this way is about 30 % of the initial dry length.

TABLE 1. SUPERCONTRACTION OF ORIENTED MYOSIN IN CAUSTIC SODA SOLUTIONS

A. Strength of caustic soda solution (%). B. Percentage supercontraction of oriented myosin from initial wet length to final wet length after washing. C. Percentage supercontraction from initial dry length to final dry length.

A	0·01	0·02	0·03	0·04	0·05	0·1	0·15	0·2	0·25	0·5	0·75	1·0	1·5	2·0	2·5	3·0
B	4	9	13	15	19	25	25	25½	25	25½	27½	27½	24	24	24	26
C	3½	8	11½	14	21	29	29½	30½	28½	28	31	27	23	23	25	25

Myosin film still disoriented in one plane behaves similarly when treated with dilute caustic soda, but of course smaller supercontractions are obtained than those quoted in Table 1 for fully oriented myosin. In one experiment, carried out on the surface of mercury, 5 min. treatment with 1 % solution resulted, after washing and drying, in a supercontraction of about 23 %.

The film used for this experiment was afterwards rewetted and immersed for 3 min. in water near boiling, transferred to cold water, and finally redried on the mercury surface. The resulting total supercontraction was about 50 % of the dry dimensions before treatment with caustic soda.

Two similar composite experiments with oriented strip gave total supercontractions of 61½ and 65½ %, respectively!

DISCUSSION AND CONCLUDING REMARKS

From the results of the experiments just described it seems impossible not to invoke intramolecular folding if we wish to explain quantitatively the whole range of contraction possible to myosin and muscle: at the very least, whether it be accepted or not that such a mechanism operates over the range of smaller contractions, it must be conceded that the myosin chains *can* fold beyond whatever state of folding is implied by the α-form, otherwise we could hardly obtain supercontractions of the magnitude quoted above. The two relevant expressions (I; Preston 1933; Astbury 1933*c*) for the elongation produced on orienting particles that are very long and thin are

$$E = \left(\frac{\phi}{\sin\phi} - 1\right), \tag{1}$$

and

$$E = \frac{(1-\cos\phi)}{(1+\cos\phi)}, \tag{2}$$

where (1) refers to an initial disorientation over an angle ϕ in one plane only, and (2) refers to an initial disorientation in all azimuths to the direction of extension. (1) shows that when the particles have random dispersion in one plane only, the maximum possible elongation by orientation is $(\frac{1}{2}\pi - 1)$, or about 57 %; while (2) shows that when they have completely random dispersion, the maximum possible elongation is 1, or 100 %. Otherwise expressed, the complete disorientation of long thin particles already disoriented in one plane only can result in a contraction of no more than about 21½ % of the initial length, and the complete disorientation of fully oriented particles can result in a contraction of no more than 50 % of the initial length.

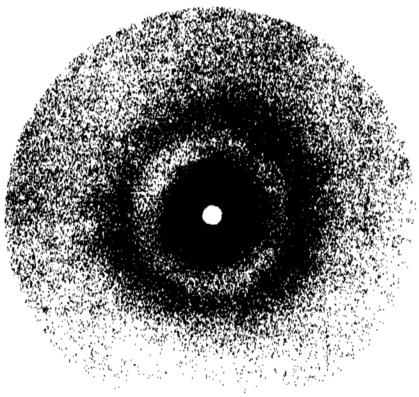
Hot water does in fact cause supercontractions approaching these amounts, and therefore it might be held that such experiments are still inconclusive; but in answer to this there are three main arguments: (1) that the X-ray and elastic properties of keratin and myosin over their *normal* range of extension and contraction cannot be interpreted except in terms of intramolecular unfolding and refolding; (2) that neither the supercontraction of keratin and myosin, nor the contraction of muscle (as will be shown in the next paper), is accompanied by such disorientation of the X-ray pattern as would account quantitatively for the observed changes in length; and (3) that with both myosin and muscle greater contractions may be brought about than are theoretically possible on the hypothesis of simply disorienting long thin particles.

But then it might be asked why the supercontracted form does not give rise to still a third X-ray fibre pattern. There is no convincing reason why it should. When elastoidin or tendon, for instance, contracts in hot water (sometimes to less than one-fifth of its initial length), the fibre photograph (collagen type) gives place to two 'amorphous' haloes (Champetier and Fauré-Fremiet 1937; Astbury 1938*a*, 1940): obviously there is folding of the polypeptide chains, but the power of forming bundles to a regular pattern is temporarily lost. And there is nothing remarkable in this: complicated dye molecules, for example, often experience a similar difficulty in building up true crystals, and it is well known in structure analysis how easily an X-ray diagram may deteriorate as a consequence of intermolecular irregularities. We do not know whether the development of further folds in keratin and myosin takes place in such a way as to give always the same new configuration, or whether there is a choice of new configurations; but even in favourable circumstances we ought not to expect the newly folded chains to group themselves with anything like crystallographic precision. After all, the best X-ray photographs of protein fibres are relatively imperfect as compared with what may be obtained from ordinary crystals.

The probability is, in any case, that the additional folds do not conform uniformly to one and the same new configuration. Between the fully extended configuration and the multiple folds of the 'globular' or 'corpuscular' proteins there will presumably be many intermediate steps, and these steps must cover the region of the degenerate or denatured proteins, the long-range elasticity of which may be very considerable indeed (Astbury, Dickinson and Bailey 1935), and also include the natural elastic protein fibres. From this point of view incidentally—and the argument cannot be stressed too strongly—*the elastic properties of hair and muscle are by no means unexpected: they fit automatically into the stereochemistry of the proteins* (Astbury 1938*a*, *b*).

In the more advanced stages of supercontraction, both of keratin and myosin, there appears a disoriented β-photograph. This is an example of the general mechanism of protein denaturation (Astbury, Dickinson and Bailey 1935). In general, when we heat a corpuscular protein there is a liberation of polypeptide chains from their specific folded configuration—this is the essence of denaturation—and the resulting product, often highly elastic, as already mentioned, will always contain a proportion of chains in the extended, or β-, form. These aggregate into regular bundles of the structure described in II and give rise to the disoriented β-photograph which is typical of all proteins at such a stage. Keratin and myosin are no exception, for their α-folds are undoubtedly the linear prototype of the

1



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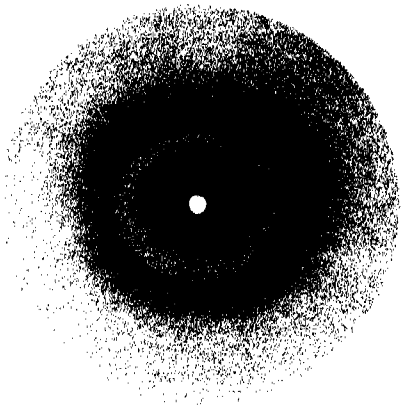

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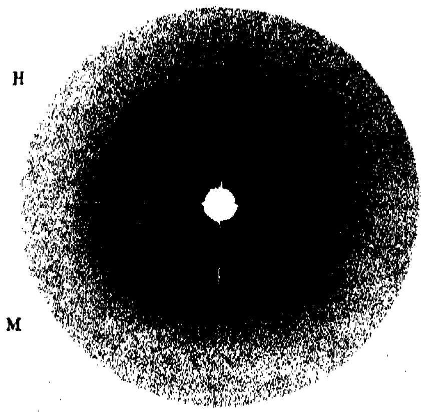
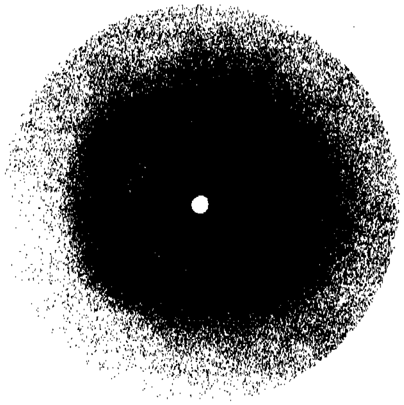


Figure 1. Myosin film, X-ray beam perpendicular to the surface.

Figure 2. Oriented α-myosin, beam perpendicular to fibre axis.

Figure 3. Exact comparison on one and the same photographic film of α-myosin (*M*) and α-keratin (horn, *H*).

4



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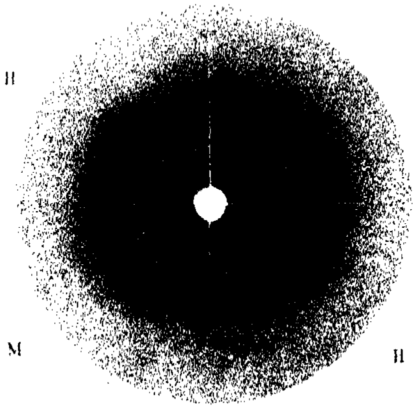

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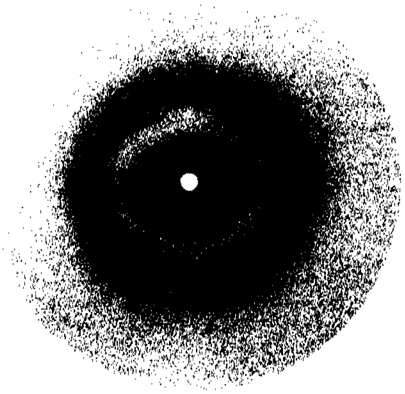
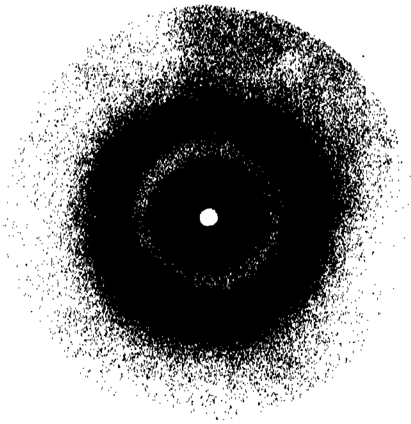


Figure 4. Oriented β-myosin, beam perpendicular to fibre axis.

Figure 5. Exact comparison on one and the same photographic film of β-myosin (*M*) and β-keratin (stretched horn, *H*).

Figure 7. Pressed myosin film, beam parallel to plane of flattening and perpendicular to fibre axis (fibre axis vertical).

8



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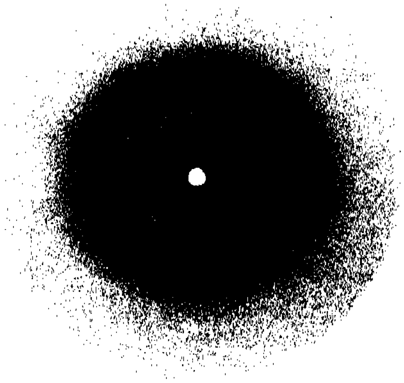

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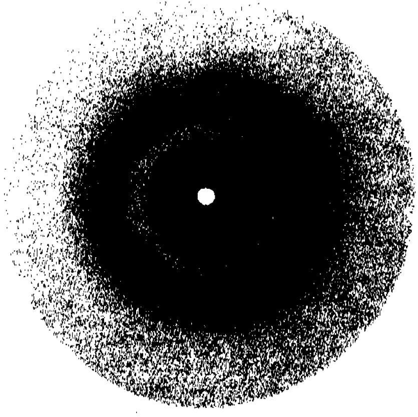

FIGURE 8. Pressed myosin film, beam perpendicular to both plane of flattening and fibre axis.

FIGURE 9. Pressed myosin film, beam parallel to fibre axis (plane of flattening vertical).

FIGURE 10. Myosin film after exposure to steam for one second, beam parallel to plane of film (plane vertical).

more generalized folds of the corpuscular proteins. Keratin requires first the breakdown of certain cross-linkages before becoming susceptible to the action of steam, but myosin passes from the α- to the β-form in water no hotter than about 55° C. In a sense this is unfortunate, but it has its compensation in that it provides an *a fortiori* demonstration of intramolecular folding; for the β-form being about twice as long as the α-form, the combined length change as a result of α-β transformation and complete disorientation should be practically zero (see above), and so actually there is even stronger reason to conclude that some of the chains at least must have become shorter than the α-form.

A concept that seems essential for the proper understanding of the elastic properties of biological fibres and similar long-chain synthetic products is that of better organized parts and less organized parts—a gradation in both size and perfection of chain-bundles or crystallites. The idea proved invaluable in interpreting the properties of keratin, but it applies with equal force to almost any biological fibre or fibrous product. Certain peculiarities of animal hairs might be ascribed to histological structure—it is sometimes argued, in spite of the convincing evidence provided by the biological cells themselves (Woods 1938), that it is uncertain from what parts of the fibre the various X-ray photographs arise; but actually these peculiarities are the consequence rather of a more general, submicroscopic heterogeneity. Whatever else is claimed from X-ray and related studies of myosin, at least they prove this great point for keratin: myosin and keratin are so amazingly similar that they stand or fall together, so to speak. And just as keratin focuses attention on the true contractile component of muscle as distinct from the aids to contraction, so myosin in turn brings the final justification for the view that the essential X-ray and elastic properties of mammalian hairs are effectively those of a single type of substance.

With all three structures, hairs, muscles or isolated myosin, we have constantly to bear in mind that there is little of the nature of true crystalline regularity, that the X-ray fibre photographs represent only the better organized parts of the structures, and that important contributions to the properties may be made by parts that for one reason or another—intramolecular penetration by water or other accessory molecules for instance—are crystallographically so imperfect as to have an all but unrecognizable effect on the diffraction patterns. The X-ray diffraction patterns must be considered only in the light of all sorts of supplementary evidence, rather as broad sketches hinting at the detailed picture. This principle was followed always in the X-ray studies of keratin already presented, and a similar line of approach is attempted here. The observed properties of keratin are consistent with the idea that the better and less organized regions are sufficiently alike to warrant the use of X-rays as a guide to the whole, provided suitable secondary experiments are carried out also; but the whole must be thought of as made up of the different parts acting *both in series and in parallel.* The better organized parts respond preferentially in such reactions as 'setting' in steam, which involves the formation of new cross-linkages (II) and is therefore favoured by perfection of aggregation; while the less organized parts function more quickly in changes requiring freedom of movement, such as the α-β transformation and supercontraction. Supercontraction is doubly elusive from the X-ray point of view, for not only is it concerned first and foremost with the less organized regions of the structure, but also, as explained above, no new strict fibre pattern ought to be expected even when the better organized regions supercontract. Nevertheless the X-ray photographs do contain the clue to what is happening, because disorientation of the α-pattern lags so much behind changes in length. The reason for this must be that at the beginning as much contraction as possible takes place in series, on account of the internal resistance to disorientation, and then, when other parts in parallel follow suit, disorientation becomes unavoidable. Finally, to supercontract the most compact regions of all requires such drastic treatment that among the products of irregular unfolding and refolding, β-crystallites are formed.

X-ray and related studies of keratin and myosin leave so little room for doubt that the two are allied in molecular configuration and elastic properties that we naturally look for resemblances also in their chemical make-up. First it must be emphasized again that *the similarity is not between myosin and normal keratin, but between myosin and the labile or supercontracting form of keratin*: side-chain breakdown must be revealed in the X-ray photographs of keratin before its properties match those of myosin completely.

This side-chain breakdown has been identified by Speakman (1933, 1934, 1936) largely with hydrolysis of the —S—S— bridges between the two halves of the cystine residues incorporated in neighbouring main-chains. Keratin has an outstandingly high and variable sulphur content, ranging from 1·35 % in porcupine quill to 5·1 % in human hair (cf. Barritt and King 1933; Speakman 1934; Bailey 1937*a*). Most of the sulphur is present as cystine, of which the yields vary from about 5 to 19 %, and only a small proportion as methionine, of which the yields vary from 0·44 to 0·67 % (Barritt 1934). Myosin however, especially mammalian and avian myosin, has a much lower, and practically constant, sulphur content, and the order of importance of the two acids is reversed, the cystine yield varying from

0·72 to 1·0%, and the methionine from 3·35 to 3·65% (Bailey 1937*a*). There seems, then, to be an obvious interpretation at least of the fact that disulphide bridges must be broken down before the elastic properties of keratin simulate those of myosin completely: it is the next best thing to not having them there at all. Looked at in this way, the hair protein may be thought of as a kind of 'vulcanized' adaptation of the muscle protein, sulphur bridges being thrown across perhaps to reduce the elastic sensitivity and increase resistance to infection and chemical attack.*

The molecules of keratin and myosin are not identical of course, but a common configuration or 'plan' must underlie them both: Nature appears to have shared the same idea between them. Indeed, it is a remarkable thing that X-rays have so far revealed only two different molecular types among the biological protein fibres (Astbury 1938*a*, *b*; 1939*a*, *b*)—the keratin-myosin type and the collagen type (Astbury 1940; Astbury and Bell 1940). And since *the former includes also the fibrils of the epidermis* (Rudall 1936; Giroud and Champetier 1936; Derksen and Heringa 1936; Derksen, Heringa and Weidinger 1937), the existence of myo-epithelial tissue, that is both skin and muscle, is almost something that might be inferred directly from the X-ray data: in fact there must be a continuous range of structures, from muscle on one hand to keratin on the other, with varying sulphur content and progressively modified elastic properties; and all must conform to a single molecular scheme.

What the minimum requirements of this scheme are we cannot yet say, at least in chemical terms, for though X-rays indicate quite definitely that the outlines of intramolecular pattern are always more or less the same, there appear to be other gross variations in amino-acid composition in passing from myosin to keratin, besides those of the sulphur acids just mentioned. The histidine : arginine : lysine ratio, for instance, is approximately 1 : 4 : 6 in myosin (Sharp 1939), while in keratin it is approximately 1 : 12 : 4 (Block and Vickery 1931; Block 1937; Block and Horwitt 1937; Astbury 1937). But it is difficult to interpret such findings at the moment: probably the answer must await complete and reliable analyses of both myosin and keratin, and more intensive research into the fine structural differences between their respective X-ray photographs. In the meantime though, there is nothing really surprising, or rather 'unorthodox', in the

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* In this connexion it is interesting to note that Linderstrøm-Lang and Duspiva (1935) and Duspiva (1936) have shown that the reason why the clothes moth can digest keratin is that it secretes a powerful reducing agent which first attacks the disulphide bridges of the 'grid': after this the main-chains are open to attack from ordinary proteolytic enzymes.

X-ray generalization that a single molecular configuration covers a range of fibres of varying chemical composition, for one of the great lessons now emerging from protein studies is that relatively few types suffice in some way for the whole gigantic field. The ultracentrifuge especially (cf. Svedberg 1937, 1939), and the stoichiometric discoveries of Bergmann and Niemann (1936, 1937*a*, *b*), suggest that only certain numbers of amino-acid residues are used in constructing the various molecular types, and surely it is not more remarkable that myosin and keratin should have a common configuration than that egg albumin, insulin, and Bence Jones protein, for example, should have similar shapes and each be built from probably 288 residues, or that fibrin and haemoglobin should both be built from 2 × 288 residues.

The Svedberg-Bergmann generalization appears to apply with equal validity both to fibrous and 'corpuscular' proteins, and it is possible to show (Astbury 1937) directly from the X-ray data that keratin almost certainly will be found to fit the scheme. The part played by the sulphur amino acids presents a peculiar difficulty, nevertheless, and the evidence rather inclines one to the view that they play a double role—that there is what may be called 'constitutional' sulphur, indispensable to the α-plan, that shows itself in the constant composition of myosin, and then 'supplementary' sulphur, that is incorporated in increasing quantities as keratin is approached. Clearly, this supplementary sulphur is not essential to the diffraction pattern and principal elastic properties, and perhaps it is embodied in a kind of cementing substance surrounding chain-bundles whose inner structure is always fairly closely the same. Similarly, the ratio of the basic amino acids cannot be the deciding factor, and as a converse example of this apparent lack of correspondence it should be pointed out that the histidine : arginine : lysine ratio of 1 : 12 : 4 has been found by Block (*loc. cit.*) for both mammalian and reptilian-avian keratin (Block regards this ratio as the distinguishing characteristic of the keratins), yet feather keratin gives a complex diffraction pattern of the β-type, instead of the usual α-pattern given by mammalian keratin (Astbury and Marwick 1932; Astbury 1933*a*, 1934, 1936).

A few concluding remarks on the denaturation of myosin may not be out of place, because this is rather a vexed question, and X-rays reveal what happens in a way not possible to chemical methods. Myosin denatures in two main steps, one associated with the α-form and the other with the β-form. Drying results in aggregation of the α-form by further interaction between the side-chains of neighbouring main-chains, but heat brings about the breakdown of the α-form and the formation of aggregates that include chain-bundles of the fully extended, or β-, type (see above). The first-mentioned step makes no *fundamental* difference to the X-ray photograph, as will be shown in the subsequent paper on the structure of muscle proper; and Mirsky (1936, 1937) has demonstrated that there is no change in the sulphur groups, as is only to be expected, since myosin is already 'configurationally disposed towards denaturation' (Astbury, Dickinson and Bailey 1935). The action of heat, however, is to disrupt the linkages of the α-grid, and then —SH groups *are* activated (Mirsky 1936, 1937), just as they are when any other naturally folded protein is so denatured. Confusion has arisen through not distinguishing between denaturation associated with the α-form and that associated with the β-form, and it is important to realize that the former involves no essential configurational disturbance, but rather only an aggregation, while the latter implies serious intramolecular breakdown also.

Occasional exceptions to the rule that drying at ordinary temperatures leaves the α-configuration substantially unchanged were observed in the early stages of this work; and in general these mishaps seemed to be associated with drying the sol too slowly, though possibly they were due to some imperfection of technique in the original extraction. At any rate, some of the films did not give a pure α-photograph: there were definite traces of the β-diagram also. It should be put on record, therefore, that the intramolecular folds of myosin can thus break down sometimes as readily as those of the sensitive corpuscular proteins.

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SUMMARY

Starting from the similarity between the X-ray diffraction patterns of muscle and myosin and those of the mammalian keratin fibres, a detailed X-ray and elastic study has been carried out of strips of myosin film prepared by drying the sol.

Methods are described of orienting the myosin chain-molecules: when oriented but unstretched they are found to give an α-photograph almost indistinguishable from that of unstretched keratin, and when stretched a β-photograph almost indistinguishable from that of stretched keratin. As with keratin the long-range elasticity rests on a reversible intramolecular

transformation, the fully extended, or β-, form of the molecule being again about twice as long as the folded, or α-, form.

The X-ray and elastic properties of myosin are found to resemble most closely, not those of natural keratin, but those of keratin that has suffered breakdown among the cross-linkages (including disulphide bridges) of the polypeptide grid. For example, by pressing moist myosin at ordinary temperatures selective orientation is brought about analogous to what is produced in keratin only at higher temperatures. Similarly, myosin supercontracts in hot water or cold dilute alkali without the preliminary stretching treatment required by keratin.

The supercontraction of myosin has been studied quantitatively and the accompanying structural changes followed by X-rays. The results show that the effect cannot be explained as due simply to disorientation of long thin units; it must involve a further folding of the polypeptide chain system.

The hypothesis is put forward that the contraction of muscle arises from supercontraction of its myosin component. It will be shown in a subsequent paper that X-ray photographs of muscle in various states, both living and dead, are entirely consistent with this view.

The fact that myosin and keratin are similar in both molecular configuration and elastic properties is discussed in the light of recent X-ray and chemical findings, and the X-ray interpretation is given of the denaturation of myosin.

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The growth, growth energy, and ageing of the chicken's heart

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The growth energy of the chicken's heart is defined, measured, and found to decline exponentially with age; it proves to be proportional to the specific growth rate of the heart *in vivo*. The growth of the heart can therefore be described by a Gompertz equation. The relation between growth, cell movement, metabolism and differentiation is discussed, as well as the relevance of the Gompertz equation to problems of relative growth and senescence.

One of the most profound convictions of the student of growth is that there is a biochemical entity called *growth metabolism* whose velocity can be measured by merely weighing an animal at intervals. The belief that the growth of animals approximates closely to the regularity and predictability with which an algebraic equation endows it can be treated as one consequence of this view. It has been justified in an insufficient number of specific instances to uphold its apparently widespread reputation for self-evidence.

A growth equation may be arrived at by purely empirical means, or it may represent a deduction from certain general theorems about growth metabolism. The merit of an equation derived by the latter method is that it can claim to have a generality wider than the sum of its instances. This merit is lost if, as is usually the case, the instances are rare and the theorems dubious.

The object of this paper is to show how, in a particular instance, a growth equation can be deduced from a body of data concerning the resistance shown by tissues of different ages to inhibition of their growth. In order that this or any other equation should be of interest to the biologist, it must be shown to satisfy certain mathematical conditions of a general nature which largely depend upon its biological context. The account of these conditions which follows will be found to supplement the well-known analysis of Gray (1929).

The biological meaning of growth equations. One important function of a growth equation is to put on record the information which is implicit in, but not made obvious by, a bald column of figures. If this were its only object, it could be fulfilled by expressing the dependence of the size (W) of a growth system upon its age (t) in the form of a power series:

$$W = k_0 + k_1 t + k_2 t^2 + k_3 t^3 + \dots$$

An expression of this sort, with an indefinite number of unassigned constants, is of very little interest to the biologist. The constants of a growth equation, upon which its meaning largely depends, may be classified into two groups according to whether one or more than one direct measurement is necessary for their specification. Those of the first group, *metrical constants*, represent magnitudes in the dimension of one of the variables; those of the second group, *velocity constants*, are dimensionless numbers whose magnitude, since they have no objective reference, can only be ascertained indirectly. A growth equation expressing the dependence of size on age should satisfy the following conditions:

(1) *The magnitude of the metrical constants must be in principle directly measurable.* If $t = 0$ represents the instant of fertilization of the ovum, then the mass of the organism when $t = 0$ is in principle ascertainable. On the other hand, the 'mass of the heart when $t = 0$' is meaningless. In practice, it is only necessary to indicate in what way an equation can be altered in order to satisfy this condition.

(2) *The magnitude of the velocity constants must be determinable by an indirect method of measurement.* This ensures that the values of the velocity constants are not *merely* those which happen to fit the data best, and that the construction of a growth equation is something more than an exercise in curve fitting. For, generally speaking, the values of the metrical constants (e.g. $W(t=0)$, $W(t\rightarrow\infty)$, $W(d^2W/dt^2=0)$) are fixed once for all by the material under investigation, and it is only the velocity constants which can be arbitrarily manipulated in order to improve the fit of the chosen equation. In this paper we shall identify the rate of decline of inhibitory resistance in the chicken's heart with the rate at which its *specific growth rate* declines. A few other students of growth have clearly realized that a condition of this sort must be fulfilled. Bertalanffy (1934, 1938) has, for instance, identified Rübner's *abnützungsquote* (mg. N excreted per kg. body weight per unit time) with the constant K of Pütter's equation:

$$\frac{dW}{dt}=HS-KW.$$

This equation states that the instantaneous growth rate of a biological system depends upon the balance between a constant multiple (H) of the absorbent surface (S) and a constant multiple (K) of the body weight (W); but the identity which Bertalanffy proposes is consistent with other formulae. Weinbach (1938) has assimilated the rate of decline of α-wave frequency in the brain to the rate at which its growth rate declines, fitting to both the equation of a monomolecular reaction. In each of these cases, then, the second of the requirements proposed above is fulfilled.

What do the points on a smooth growth curve represent? Ideally, a growth equation should predict for any chosen age a value about which the actual masses of the members of a population of organisms of that age should be normally distributed. Conventional tests for the efficiency of a growth equation depend upon the assumption that departures from the values required by it will be of this sort. But more frequently a growth equation defines only a *mean curve* of growth, and departures from it may take the form of cycles of a more or less regular sort. It is, for instance, only the annual curve of growth of the Pacific razor-clam which is described by a Gompertz equation (Weymouth, McMillin and Rich 1931); the continuous curve of growth describes a series of regular annual cycles about it.

Difficulties of a special sort arise when there are genuine discontinuities in the growth curve, i.e. when the total curve is broken up into periods each of which must be described by functions of a different sort, or by a single function whose constants abruptly change their values at the appropriate moments. When in such cases the growth curve is compiled from mass statistics, there is a real danger that curves of growth will be vitiated to a sometimes incalculable extent by curves of distribution. It should be added that the resolution of growth curves into a series of, for example, exponential cycles is very seldom justified.

The range of application of a growth equation. It is often mistakenly supposed that an equation which has described reasonably well the growth of a whole organism must apply with necessary justice to the growth of its parts or any combination of its parts. It is a fact, however, that the sums and averages of the common growth functions are not themselves functions of the same sort, unless certain very special conditions are fulfilled. There are therefore no universal growth equations other than those in which the functions are themselves ambiguous, e.g.

$$\frac{dW}{dt} = KW\,f(t). \tag{1}$$

The proper mode of expression for growth equations. The proposition 'biological growth is fundamentally of the multiplicative type' can be taken to mean that what results from biological growth is itself usually capable of growing. Unfortunately, 'multiplicative growth' is often exclusively associated with the degenerate case, $f(t) = constant$ in equation (1), of growth by the compound interest law. This limited use cannot be upheld, although it is usually convenient to treat the case of growth by the compound interest law as a norm with respect to which the various departures from it can be classified. It follows that the most appropriate form of expression for a growth equation is one evaluating the *specific growth rate*,* or rate of change of size as a function of size. The specific growth rate of a sum growing by the compound interest law is constant, and its specific acceleration zero. For typographical convenience, we shall denote by $\phi(W)$ the specific growth function of a system of size W, and make use of the identity

$$\phi(W) = \frac{1}{W}\frac{dW}{dt} = \frac{d\log_e W}{dt}.$$

Some authors have made the acceptance or rejection of this formulation a matter of principle. Clearly, however, it involves questions of expediency alone. The most important classes of estimates of $\phi(W)$ have been reviewed by Teissier (1937).

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* Following Gray (1929). The term 'relative growth rate' has come to have another meaning in biology.

From a number of determinations of size separated by equal time intervals, an approximation $\phi'(W)$ to $\phi(W)$ can be calculated from the expression

$$\phi'(W) = \frac{1}{W}\frac{\Delta W}{\Delta t},$$

whose efficiency, as Brody (1926) has shown, depends upon the magnitude of the finite time interval Δt (during which growth is proceeding continuously) in relation to the physiological age of the organism. Expressed as a percentage growth rate per unit time, $\phi'(W)$ gives an estimate *always higher* than the corresponding value for the instantaneous growth rate. It is a sensitive estimate, because it magnifies very strongly any small irregularity in an apparently smooth growth curve; and it will be vitiated by growth periodicities whose intervals do not happen to coincide with the intervals of measurement.

These and other disabilities point to the need for an *experimental measure of the specific growth rate* which can be compared with that directly calculated from the regression of size on age. The method of explantation, on the lines laid down by Cohn and Murray (1925), is clearly indicated, because the explantation of a tissue automatically isolates it from sources of variation which, while they influence growth, are by no means necessarily relevant to its study. The following data concern differences between the growth characteristics of tissues which can be regarded primarily as functions of their embryonic age:

Suzuki (1925*), and independently Cohn and Murray (1925), showed that the latent period preceding the first appearance of cells from the explanted tissue increased with the embryonic age of their source. This fact was confirmed by Oda and Kamon (1927) and Olivo and Slavich (1930*b*), and generalized by Hoffman, Goldschmidt and Doljanski (1937 *a*, *b*, *c*). The spleen (Oda and Kamon 1927) is an exception to this rule, and it is noteworthy that Trowell and Willmer (1939) find that saline extracts of embryonic spleen stimulate growth more feebly than corresponding extracts from the spleen of older animals.

Upon the length of the latent period before migration depends to some extent the magnitude of outgrowth from the explant during the first few days of cultivation; but these initial age differences rapidly smooth out. Strains of fibroblasts differing only in the embryonic age of the donor tissues rapidly reach uniformity to stimulation (Hoffman *et al.* 1937*a*) or show constant racial differences not obviously correlated with their initial embryonic age (Parker 1933).

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* For this reference, I am indebted to Needham's *Chemical Embryology* (Cambridge, 1932).

Suzuki showed that the threshold of susceptibility of chick heart fibroblasts to diphtheric toxin declined as the embryonic age of the explant increased. It is interesting that older *embryos* are on the whole more refractory than young to the generalized lethal effects of diphtheric toxin introduced through the chorio-allantoic membrane (Evans 1938).

Experiments of the converse type, in which the age of the source of the cultivation medium rather than the age of the tissue is made the independent variable have been undertaken by Gaillard (1935) and Fowler (1937). They do not reveal the uniform decline in the growth-stimulating power of tissue extracts which might have been expected; generally speaking, 'embryo extract' is more potent when prepared from embryos of 10–20 days age than from embryos younger than 10 days.

None of the data recorded in these papers are amenable to an exhaustive analytical treatment; nor were they intended to be. They do not therefore provide us with the information we require.

The first method considered as a possible experimental measure of the specific growth rate was the determination of the relative growth increment of tissues of different ages grown in a medium such as blood plasma which is capable of supporting life without promoting growth. Willmer's apparatus (Willmer and Jacoby 1936) can make direct measurements of this sort fairly accurately, but it is designed for intensive rather than extensive analysis. The second was to subject each explant to a standard quantum of inhibitory substance in order to keep the total amount of growth down and to magnify small absolute differences in growth magnitude. It was found that this merely reproduced on an insufficiently small scale the disabilities of the method of direct measurement. Instead, we have determined the concentration of an inhibitory substance *just sufficiently high* to inhibit all outgrowth from a series of explants of differing embryonic ages under strictly uniform conditions of cultivation. The magnitude of this threshold for total growth inhibition, measured in arbitrary units, will be described as the *growth energy* of the tissue in question. The concept of growth energy itself, and much of what is subsequently deduced from it, depends upon the choice of this third method for examining the distribution of growth characteristics in time.

The heart of the embryonic chick was an obvious choice as experimental material. Its behaviour *in vitro* has been well documented; it consists entirely of mesenchymal elements; and its growth is not markedly anisomorphic from the sixth day of embryonic life onwards. Furthermore, determinations of its mass have been made by Schmalhausen (1926) over the age period relevant to these experiments. The *inhibiting factor* is that discovered by Heaton (1926) and reported on by Heaton (1929) and Medawar (1937). It is a water-soluble, surface-stable and thermo-stable (100° C) substance possessing the remarkable but not unique property of inhibiting, at the appropriate concentration, only the growth of mesenchymal elements in a mixed tissue. It is not toxic 'in any non-specific sense'. Its various properties, described in my former paper, are such as to render it suitable for an investigation of this sort.

TECHNICAL METHODS AND EXPERIMENTAL DATA

The method of determining inhibitory thresholds will be called the *assay method*, because it is identical in principle with that used for determining the strength of the inhibitory factor itself, with respect to its action on 10-day-old heart explants (Medawar 1937). In practice it differs from it because the age of the tissue, rather than the concentration of the inhibitor, is made the independent variable. The assay method takes advantage of the fact that small differences in the absolute magnitude of growth are easily discernible when the total amount is very small.

In the majority of the experiments described below, the inhibitory factor has been prepared by one of the methods described in my former paper; but each stock solution is now subjected to a routine partial decolorization in purified animal charcoal (Merck), to remove surface active matter and water-insoluble residues. In other experiments I have been able to use considerably purified media prepared by Lady Robinson.

A number of small changes have been made in the assay technique:

The solution of the over-strength inhibitory factor in saline is prepared by the methods previously described, but the dilutions prepared from it now approximate to a logarithmic series (1:1; 9:10; 8·1:10; 7·3:10; ...). From each level of this *dilution series* approximately 0·3 ml. is removed and diluted, in a further set of tubes, with a precisely equal volume of fresh chicken plasma prepared by Bordet's method, without heparin. These constitute the *media series*. From each level of these, five or more 0·1 ml. samples are measured with a graduated capillary pipette into the wells of a corresponding number of uniform hollow-ground slides and spread out over a circle of 2 cm. diameter.

Into each is now rapidly introduced an even fragment (approximately $1\frac{1}{2}$ mm.^{3}) from the outer walls of the ventricles of the heart of a 10-day-old chicken embryo. The use of even a trace of coagulant has been abandoned.

After 24 hr. incubation at 39° C, the level in the *culture series* which corresponds to inhibitory threshold can be determined by a rapid inspection.

One unit is defined as the amount of inhibitor *sufficient and necessary* to inhibit all outgrowth from one standard 10-day-old explant under precisely the conditions which have just been described. Since 20 culture cells contain 1·00 ml. of a member of one of the dilution series, the member in question is said to contain 20 units per ml. It has been found most convenient to refer the inhibitory threshold of explanted tissues to the concentration of the inhibiting substance in the dilution series. The magnitude of the growth energy of 10-day-old explants is therefore *defined* at 20 units.

Once the strength of the inhibitory medium has been determined, it should only be necessary to replace the standard 10-day-old tissue with one of another age, whose growth energy could then be determined by a direct reading of the inhibitor concentration. Unfortunately, the growth energy of 10-day-old tissues, to which a constant and arbitrary value of 20 units is assigned, must itself be regarded as variable. In order that errors due to this otherwise concealed variation of the standard tissue should be normally distributed through the readings for other ages, the 10-day-old tissue has in each instance been cultured side by side with the tissue whose growth energy requires to be determined. Each experiment thus constitutes its own assay for the inhibitory factor. Errors due to the variation of the standard tissue are further discounted by the large number of explants which have to be used. Not less than five 6-day, three 8-day, and two 10-day-old embryos are needed to supply the appropriate number of explants in each instance.

Rapidity and uniformity of technique has been made possible by a drastic modification of normal tissue culture apparatus. The dilution tubes are supported and covered by glass capsules held firmly in a brass rack. The slides are secured face upwards in brass frames covered by a single glass sheet. The use of the well of the slide as a culture cell is for our purposes in every way preferable to the use of a cover-slip; and it has been found that the cultivation of two explants instead of one in each cell has no effect on the magnitude of the inhibitory resistance of either which is detectable by the assay method.

The validity of the assay method in the experiments we have described clearly depends upon the uniformity in growth properties of the walls of the ventricles of the heart. It is important, therefore, that any heterogeneity—whether or no in the form of a gradient of resistance—should not be detectable by the methods which show up strongly a gradient of inhibitory resistance in time. If a set of hearts is dissociated in any regular way (figure 1) and explanted into a medium containing the inhibitory factor just below threshold strength, it can easily be shown that the growth magnitudes of the ventricular elements are sufficiently even to justify a hypothesis of uniformity. On the other hand, the arterial arches are extremely resistant to growth inhibition, and the auricles more resistant than the walls of the ventricles. With these facts we are not for the present concerned.

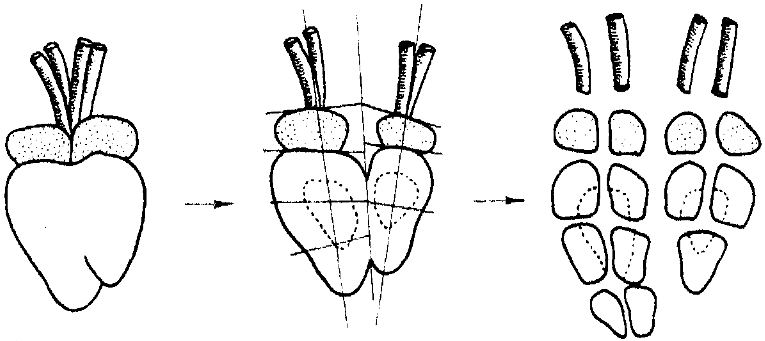

FIGURE 1. The ventricles of the heart are incompletely separated by a sagittal incision and cut transversely. The pieces are then explanted into numbered cells.

For various reasons, the assay method it not adaptable for use over a range greater than 6–18 days; nor is it sufficiently subtle to reveal, within a practicable number of determinations, differences between the growth energies of tissues differing by only a day in age. The results of seventy-five experiments, entailing the examination of rather more than 4000 cultures performed under quantitative conditions, are therefore tabulated below for tissues of 6, 8, 10, 12, 14, 16 and 18 days only.

A representative experiment (37) illustrates how the threshold point for total inhibition is recorded in a particular instance, and how the data are written up for future reference.

After 24 hr. incubation, the cultures are removed and inspected. The explants from the 10-day-old and 16-day-old hearts lie side by side in each cell, and the extent of outgrowth from each is recorded. 10-day threshold point is taken at series 2, in spite of the anomalous result in cell 5; and 16-day threshold point in series 4. Accordingly the growth energy of 16-day-old tissues from this experiment, corrected to the nearest quarter unit, is given by

$$20 \times \frac{0{\cdot}73}{0{\cdot}90} = 16{\cdot}25,$$

since the threshold point for 10-day tissues is *defined* as 20. Thirteen other such readings, as the table indicates, provide the mean point for 16-day tissues upon which the analysis is based.

EXP. 37. 16-DAY/10-DAY

Dilution* series	ml.	Culture series	Cell	10-day	16-day
1	1·00	1	1	No growth	No growth
2	0·90		2	—	—
3	0·81		3	—	—
4	0·73		4	—	—
5	0·66		5	—	—
6	0·59	2	1	Free growth	No growth
7	0·53		2	—	—
8	0·48		3	—	—
			4	—	—
			5	No growth	—
		3	1	Free growth	No growth
			2	—	—
			3	—	—
			4	—	—
			5	—	Cell processes
		4	1	Free growth	Marginal growth
			2	—	—
			3	—	Cell processes
			4	—	Cell processes
			5	—	Free growth
		5–8	Free growth throughout in both sets		

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* The *dilution series* represents parts of inhibitor solution in saline in 1·00 ml. saline. The *media series* are prepared by diluting 0·3 ml. from each member of the former with 0·3 ml. plasma, yielding eight sets as before. The *culture series*, each member consisting of five culture cells, represents five 0·10 ml. samples from each member of the media series.

In other examples, the threshold point is judged to lie *between* two series, as it does in the following instance:

Culture series	Cell	
3	1	No growth
	2	Cell processes
	3	—
	4	No growth
	5	—
4	1	Cell processes
	2	Free growth
	3	—
	4	—
	5	—

TABLE OF GROWTH ENERGY OF CHICKEN'S HEART

Age in days	No. of exps.	Geometric mean of growth energy	Standard error	Calculated value	Difference
6	16	1·3861 (24·33)	0·0069	1·3869 (24·37)	+0·04
7				1·3662 (23·24)	
8	15	1·3476 (22·26)	0·0081	1·3455 (22·16)	−0·10
9				1·3248 (21·12)	
10	—	1·3010 (20·00)	—	1·3041 (20·14)	+0·14
11				1·2834 (19·20)	
12	7	1·2686 (18·56)	0·0064	1·2627 (18·31)	−0·25
13				1·2420 (17·46)	
14	15	1·2141 (16·37)	0·0085	1·2214 (16·65)	+0·28
15				1·2007 (15·87)	
16	14	1·1832 (15·25)	0·0064	1·1800 (15·14)	−0·11
17				1·1593 (14·43)	
18	8	1·1385 (13·76)	0·0098	1·1386 (13·76)	0·00

The figures in brackets are the antilogarithms, corrected to two decimal places, of the corresponding figures in the previous column. It is to the former that the column of differences refers.

The *calculated values* are those required by the equation

$$\log GE = \log A - k't, \tag{2}$$

where GE = growth energy, $\log A = 1{\cdot}510976$, $k' = 0{\cdot}020688$ ($\pm 0{\cdot}000444$). The constant k' represents *half* the calculated linear regression coefficient of $\log GE$ on age, since the measurements were taken at two-daily intervals. The appropriate adjustment has been made in the figure representing its standard error.

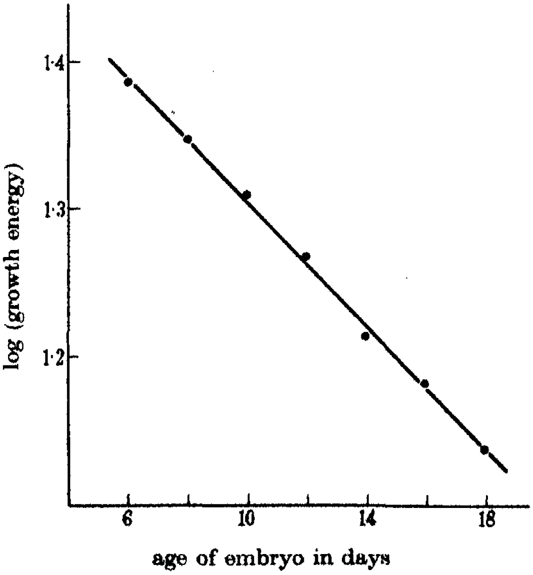

FIGURE 2. The logarithm of the growth energy of the heart as a linear function of its age. The regression line is that calculated from equation (2) in the text.

EXPLANATION AND ANALYSIS OF DATA

Equation (2) records the assumption that the logarithm of the growth energy of the heart declines as a linear function of its age. A comparison of the variance within arrays with the variance due to regression gives a value of $e^{2z} = 2{\cdot}537$, corresponding to about a 20 % point. The assumption is therefore justified, as the close correspondence between the actual and calculated figures for the growth energy suggests. The growth energy itself therefore declines exponentially, according to the equation:

$$GE = Ae^{-kt}, \tag{3}$$

where $k = 2{\cdot}3026k'$, t = age of tissue in days, $A = GE\ (t=0) = 34{\cdot}42$ (corrected to two places of decimals).

The efficiency of the assay technique is shown by the significance of the difference between the two arrays of readings whose means lie closest together, viz. 14-day = 1·2141, 16-day = 1·1832. For the 27 degrees of freedom contributed by the two arrays, $t = 2{\cdot}873$. For $P = 0{\cdot}05$, $t = 2{\cdot}052$. The two sets of readings cannot therefore be reasonably regarded as samples from the same normal population.

The use of the geometric mean is justified by the fact that the frequency distribution of the logarithms of the separate values is more nearly normal than the corresponding distribution of the arithmetic values. The skewness of the distribution of the latter is naturally most obvious in the readings for the younger tissues; the difference between the two means in tissues older than 12 days is very slight.

Interesting statistical problems are raised by the fact that the growth energy of 10-day tissues is *defined* as 20, while its calculated value is 20·12. This has no effect on our subsequent reasoning, in which we are concerned with the *rate of decline* of growth energy with age, and in no case with its actual values. The figure 20·12 happens to correspond to the best straight line through the logarithms of the observed values. For much the same reason, it is of no importance that the limiting value of the growth energy is given by (3) as zero. The subtraction of a further metrical constant in the dimension of t can be made to rescue the constant A from representing the fictitious value of the growth energy of the heart when $t = 0$.

Four-day-old hearts have been excluded from this analysis for several reasons. The fact that an explant of suitable size consists of at least half the ventricle, and that it accordingly tends to undergo 'organized growth', makes it unsuitable for an analysis of this sort. It has been our experience that the growth of the 4-day-old heart in a plasma-saline medium is initially feeble; but that its growth energy, although variable, is usually higher than that of 6-day-old hearts. This illustrates the distinction which the assay method allows us to draw between the growth energy of a tissue and its specific intensity of growth.

Deduction of a growth equation for the heart

We are now in a position to investigate the natural assumption that the decline of the growth energy of the heart as its age increases is a measure of the rate at which its specific growth rate declines *in vivo*. If this is the case, then

$$GE = K\phi(W),$$

where W represents the mass of the heart; and from (3)

$$\frac{d \log_e W}{dt} = \frac{A}{K} e^{-kt},$$

$$\log_e W = b - ae^{-kt}, \quad a = A/Kk, \tag{4}$$

$$W = W_i e^{-ae^{-kt}}, \quad W_i = e^b. \tag{5}$$

Here $W_i = W$ $(t \to \infty)$, and $W_i e^{-a} = W$ $(t = 0)$. This Gompertz equation asserts that the heart of the chick grows *at a rate of continuous compound interest which itself declines by continuous compound interest.*

We have now to consider the possibility that the growth energy of the heart is a measure of some characteristic which, while distributed exponentially in time, is not proportional to and therefore not a measure of the specific growth rate of the heart *in vivo*. It might very well be one of those characteristics, like the rate of egg-laying in chickens or the rate of healing of wounds (as functions of age), which decline by compound interest (Brody 1928), and whose sum is sometimes held to constitute the phenomenon of 'senescence'. We shall use two methods to establish the identity we propose.

(1) Let $w_0, w_1, w_2, \ldots, w_t$ be the common logarithms of successive and equally spaced values of the masses of a growth system dependent upon a Gompertz function of time. If D be the series of differences between successive logarithms, such that

$$D_1 = w_1 - w_0,$$

$$D_2 = w_2 - w_1,$$

etc.

then the coefficient of the regression of log D on time, multiplied by 2·3026 to correct it to natural logarithms, is an estimate of the constant in the

Gompertz equation (k in equation (5)) which represents the rate of decline of the specific growth rate.

If our hypothesis is correct, then the value of k (or k') calculated from the growth energy of the heart, and that calculated by the method just described from the figures of Schmalhausen (1926), should not be significantly different. The former estimate gave us $k' = 0{\cdot}020688$ ($\pm 0{\cdot}000444$); the latter gives us 0·02396. The latter figure is unfortunately not significantly different from zero; the construction of a Minot curve from Schmalhausen's data:

$$\%\ \text{growth rate} = \frac{100(W_{t+1} - W_t)}{W_t},$$

reveals a very strong periodicity in the growth rate of the heart (cf. also figure 4) which is such as to vitiate the method we have described for estimating the rate of decline of the specific growth rate. The figures are however suggestive. From the data of Olivo and Slavich (1930*a*), based upon protocols less complete than those of Schmalhausen, we find $k' = 0{\cdot}02593$. Olivo and Slavich have also calculated the *mitotic coefficient* (fraction of cells in the field seen to be undergoing mitosis, × 1000) of the heart *in vivo* at different ages. They find it to be a measure of the percentage growth rate of the heart, calculated from their own figures; but the decline in the mitotic coefficient bears no simple relationship to our own data for growth energy, and is not exponential.

(2) Alternatively, we may fall back on graphical methods, and re-express equation (4) in the form

$$\log_\epsilon W = b - \frac{\phi(W)}{k},$$

i.e. there should be a linear relation between the logarithm of the mass of the heart and its specific growth rate for corresponding ages. This corrects the logarithmic growth curve back to the straight line which is characteristic of a constant specific growth rate, by making allowance for the degree to which it actually departs from it. Accordingly, our hypothesis may be tested by plotting the logarithms of successive masses against the *growth energy* for corresponding ages. This test takes two forms. In figure 3 they are plotted against the *observed* values, in figure 4 against the *calculated* values of the growth energy. Both appear to be adequate linear regressions, although they cannot be critically analysed since Schmalhausen did not publish the appropriate protocols. On the whole (3) is better than (4), because any marked fluctuation in the specific growth rate might well be reflected in the observed figures for growth energy, notwithstanding the fact that the best simple algebraic curve relating the points is exponential.

The lines of evidence we have considered entitle us to come to the following conclusions:

(1) The growth energy of the heart is a negative exponential function of time over the range 6–18 days.

(2) The growth energy of the heart is proportional to, and therefore a measure of, its specific growth rate *in vivo*.

(3) The mass of the heart can therefore be said to be dependent upon a Gompertz function of time over the age period under consideration.

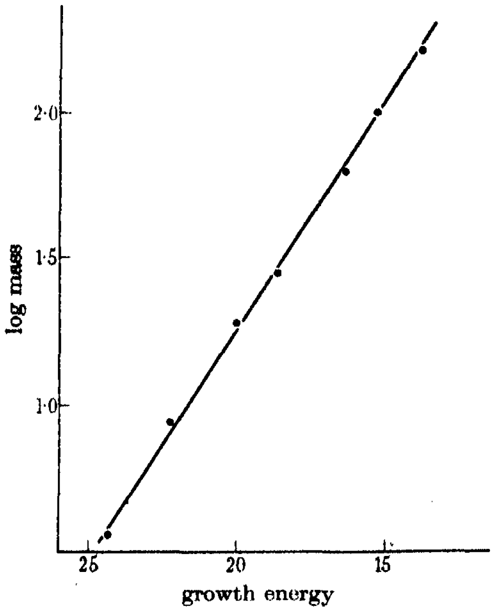

FIGURE 3

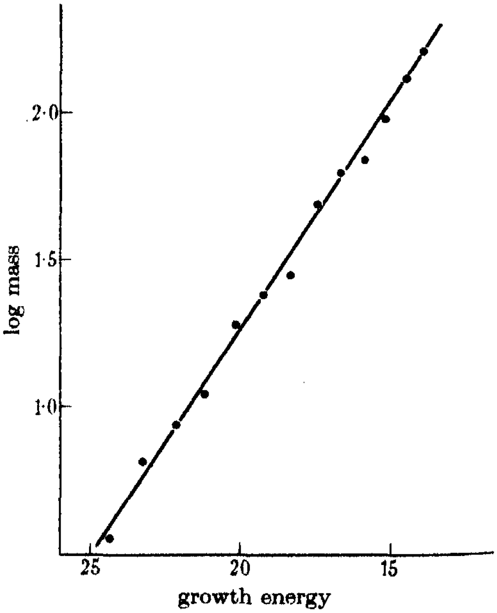

FIGURE 4

FIGURE 3. The logarithm of the mass of the heart (ordinates: figures of Schmalhausen (1926), × 1000) as a linear function of its growth energy (abscissa: antilogarithms of the geometric means from the Table of Growth Energy).

FIGURE 4. Cf. figure 3. In this case the abscissa represents the antilogarithms of the *calculated values* of the growth energy. The periodicity referred to in the text is obvious.

We have therefore shown that the most important velocity constant of a growth equation, that measuring the rate of decline of the specific growth rate and responsible for the flattening of the curve, can be evaluated by indirect and (as it happens) experimental means. We have now to consider with what department of metabolism the growth energy of a tissue can be supposed to be associated.

Further evidence relating to 'growth energy' and its interpretation

The *growth energy* of the heart has been described as a measure, in arbitrary units, of the magnitude of its resistance to a chemical substance inhibiting its growth. The growth energy declines in a regular way as the heart grows older.

Superficially, the cultivation of an explanted tissue might be expected to *lower* its resistance to growth inhibition, by dissipating its well-known power of proliferating for short periods in incompletely nutrient media. The opposite is the case. By using the assay method to compare fresh explants of an assigned embryonic age with others of the same age which have been cultivated for one or more passages in the control plasma-saline medium, it has been found that the decline of growth energy with increasing embryonic age *is a property of the freshly explanted tissue only.* During the course of cultivation the threshold for total inhibition *rises* sharply; and in the course of a fortnight in the case of younger tissues reaches a virtually constant value. It is furthermore the case that the initial differences between tissues from donors of different ages rapidly smooth out during cultivation; after 2 weeks, it is no longer possible to distinguish by means of the assay method between explants originating from 6-day-old and 12-day-old embryos.* This is consistent with the findings of Hoffman *et al.* (1937*a*) and Parker (1933), which were referred to earlier.

The increase of resistance to inhibition during the course of cultivation is strongly accelerated by the presence of embryo extract, though it is not uniquely dependent upon it. Embryo extract is strongly antagonistic to the action of the inhibitor, even in primary cultures. Extracts from 10-day-old embryos at a final concentration of 12½ % in the culture medium raise the inhibitory threshold for freshly explanted 10-day-old tissues by about 25 %—thus assimilating their response to that of tissues only 6 days old. The effect of embryo extract during cultivation is not however merely an immediate one. The inhibitory resistance of parallel strains, one of which has been cultivated in embryo extract and the other in the control plasma-saline medium, can still be distinguished *even when embryo extract is intro-*

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* The curve relating the rise of growth energy to the time of cultivation (t') may very well be the inverse of that representing its decline with ageing *in vivo*, i.e.

$$GE_{\text{exp.}} = GE_t - (GE_t - GE_0)\, e^{-kt'},$$

where $GE_{\text{exp.}}$ is the growth energy of the explanted tissues; GE_0 the initial value recorded in the experiments of the preceding section, and GE_t the constant limiting value ultimately assumed by tissues of all ages after lengthy cultivation. The constant k may well have the value previously assigned to it.

duced at saturation strength into the media in which they are finally assayed. This points to some cumulative action on the part of embryo extract which is consistent with the view that it accelerates whatever change in the metabolism of tissues normally occurs during cultivation. The nature of this change is considered below.

We are now in a position to account for the facts so far presented in this paper. They are not consistent with Parker's hypothesis (1933), held by many tissue culturists, that differences of the sort we have described are due to the steady increase with ageing *in vivo* in the density of intercellular matrices. The assay method determines in each instance the resistance of the *most resistant* cells in each explant. If 6-day-old and 18-day-old explants differed only in the resistance which the intercellular matrix offered to the migration of otherwise equipotent cells, we should expect to find a marked difference in the *density* of growth at equal inhibitory concentrations, but no necessary difference between the thresholds for total growth inhibition. For it would merely happen that one cell in every three, say, would be prevented from leaving the explant. We find, on the contrary, that the growth energy of 6-day-old tissues is nearly twice as high as that of 18-day-old tissues.

For the same reason precisely, the *rise* of inhibitory threshold during cultivation cannot be due to the progressive selection of the most resistant cells; nor could such a selection operate in one or two passages. It is sometimes said that effects similar in principle to the ones we have described here are due to the 'adaptation' of tissues to a life *in vitro*; but this is merely a restatement of the empirical facts in a new way.

In the absence of biochemical evidence of other than a negative nature, we are obliged to rely on further circumstantial evidence relating to growth and growth energy. The term *growth* energy itself is only justified by the correlation subsequently established between the phenomenon it denotes and growth. The measure of this phenomenon depends, however, on the ability of cells to *migrate* from the central explant in the presence of a chemical factor whose depressant action on growth may not be brought about directly. We shall now give evidence to show that the *multiplication* of mesenchyme cells, which is a limiting factor for the increase of tissues in mass, is itself dependent upon their movement; and that it is in the department of metabolism associated with cellular movement that we must look for the physiological basis of growth energy and the phenomena shown to be correlated with it.

Part of a paper by Jacoby, Trowell and Willmer (1937) dealt with the relation between the duration of the ana-telophase period of mitosis and the intensity of cellular movement in fibroblasts. The data in question, part of which were collected by myself, have been statistically re-examined. They suggest very strongly that the mechanism by which fibroblasts draw themselves apart at the conclusion of mitosis is of the normal migratory type—as the direct observation of the process in cinematograph films strongly indicates. The inhibition of cellular movement in fibroblasts must therefore entail, as a secondary consequence, the inhibition of cell multiplication. It is commonly agreed however that in epithelial tissues, and in the cleavage of developing eggs, the energy for cytokinesis does *not* come from ordinary amoeboid movement; nor is cell division in epithelia necessarily accompanied by it. Factors inhibiting the growth of fibroblasts through the medium of cell movement should not therefore affect the multiplication of epithelia at concentrations at which they are fully operative on the former.

There are three well-known groups of chemical inhibitors which are known to inhibit reversibly the growth of fibroblasts. These are (1) the group of which the inhibitor under consideration in this paper may be the only representative; (2) members of the group of aldehydes whose properties have been investigated by Pomerat and Willmer (1939); and (3) the group of surface-active substances examined by Katzenstein and Knake (1931). Inhibition by agents of all three groups has the same initial cytological effect on fibroblast cells, which round off, i.e. return to the position of minimal surface tension which must result from the inactivation of the source of energy which is normally responsible for distorting them into amoeboid shapes. Surface-active substances probably bring about this effect by direct accumulation on the cell surface; inhibitors of the other groups are not markedly surface active.

If members of any of these groups of inhibitors directly affected mitosis or the synthesis of cytoplasmic substance, they should act with equal vigour on epithelial tissues. This is not the case. Neither the surface-active substances (Katzenstein and Knake 1931) nor the inhibitor under consideration here (Heaton 1926; Medawar 1937) affect the growth of epithelia at concentrations at which they totally inhibit the growth of fibroblasts. It is also noteworthy that the cleavage of the ova of *Urechis* and *Strongylocentrotus* is not inhibited (Ellis 1933) by a concentration of iodoacetate 200 times stronger than that necessary to inhibit the growth of fibroblasts (Pomerat and Willmer 1939).

There remains to be considered the group of aldehydes.

The threshold strength for inhibition by propionaldehyde, according to the assay technique, is that which yields in the culture medium a *final* concentration of $M/150$. It is slightly preferential to the growth of epithelia, which have been grown pure or almost pure on the surface of coagula containing final concentrations of propionaldehyde ranging from $M/20 \rightarrow M/100$, according to the age and source of the donor tissue. It is very probable that pure aldehydes are not satisfactory media for exerting differential effects of this sort, owing to their considerable secondary reactivity. Preliminary experiments by Lady Robinson are consistent with the view that the active group of Heaton's inhibitor is aldehydic, while the substance as a whole has the carbohydrate attachments which its solubilities suggest (Medawar 1937).

The original observation of the inhibitory action of aldehydes came about in the following way. One method of preparing a crude stock solution of Heaton's inhibitor is to concentrate an alcoholic extract of malt by partitioning with ether, the lower layer being further concentrated by evacuation on a water bath. If impure ether is used, its peroxides oxidize the alcohol to acetaldehyde which, owing to its polymerization, cannot be completely removed by evacuation. The residue exerts differential effects comparable with those of the inhibitor itself.

It is nevertheless clear that in the three instances we have given above, the differential action on epithelia and the cytological action on fibroblasts strongly indicate that the inhibitory effect on the multiplication of mesenchyme cells is brought about by the inhibition of their movement.

It is not yet possible to make any confident statement about the department of metabolism associated with cell movement and growth in fibroblasts. Pomerat and Willmer have suggested that the growth of fibroblasts is intimately connected with direct glucose breakdown, i.e. without the cycle of phosphorylation that is characteristic of skeletal muscle. Although a skeletal muscle cycle has been recorded in tumour (Boyland, Boyland and Greville 1937), brain (Meyerhof 1938) and embryo (Meyerhof and Perdigon 1939), it is stated that there is no phosphorylation of glycogen in chicken embryos younger than 18 days (Gill and Lehmann, in a personal communication). Needham and Nowinsky (1937) and Needham and Lehmann (1937) have for some time maintained that direct glucose breakdown is characteristic of avian embryonic tissues. The view that the decline of growth energy with age is an index of the decline in the specific intensity of glycolysis would therefore imply the acceptance of one of two highly controversial views on the nature of embryo carbohydrate metabolism. Whatever change in type or degree of metabolic activity accompanies development is very probably reversed by cultivation; and it is also very probable that this is the only sense in which cultivated tissues revert to an embryonic type. Laser (1933) has, for instance, shown that a preliminary period of 'adaptation' *in vitro* is necessary before fibroblasts can subsist anaerobically. But against any attempt to associate carbohydrate metabolism with growth, there is the clear demonstration by Boyland and Boyland (1939) that there is no positive correlation between the growth rate of tumours and the intensity of their glycolysis.

It is not therefore possible to adhere to any particular interpretation of the precise mechanism by which the growth energy of cells and the specific growth rate of tissues decline as they increase in age. The dependence of cell multiplication on cell movement in fibroblasts and other mesenchyme cells shows that the growth of mesenchyme tissues at least must be envisaged in terms of their *topological differentiation*; i.e. with the sort of gross morphological differentiation which continues long after the assignment of histological type, and which is chiefly concerned with the stretching, folding, and also the amplification of surfaces.* Cell movement must be primarily responsible for differentiation of this sort in organs like the heart, with differential growth secondarily dependent upon it. The Belgian embryologists (Pasteels 1937; Dalcq 1938) have already shown the close relationship between cell-movement (rather than, for example, relative cleavage rates) and topological differentiation in the early embryo. A close study of the metabolism of the embryonic heart might therefore provide the basis for the first intimate correlation between the growth, movement and metabolism of cells, and the topological differentiation of tissues; and might be able to indicate the degree of their functional dependence upon one another.

The Gompertz equation in relation to relative growth and senescence

The evidence collected together in this paper, although independent of any particular construction that is put upon it, suggests strongly that over the range 6–18 days—equivalent in 'physiological time' to a high proportion of the life cycle—the growth in mass of the chicken's heart is described by a *mean curve* which is that of a Gompertz equation. What the evidence does *not* permit is the generalization of this function to cover the entire range of growth processes in plants and animals. This reservation, mild as it may seem, does much to destroy the interest of the biologist in attempts to describe the course of growth mathematically; although, as we have seen, the impossibility of explicit universal growth equations is not due to the intractability of living organisms, but simply to the mathematical properties of the functions commonly used to describe their growth. It was not therefore the object of the paper to establish any *particular* growth equation. It was partly to indicate the lines upon which the analysis of the relation between growth, movement, metabolism and differentiation might profitably be attacked; and partly to show that a growth equation can be deduced from theorems expressing a number of physiological *facts* about growth. Theorems such as these must eventually replace the ones which have often been made the premises for reasoning of this sort; which, for instance, assimilate the rate of growth to the rate of chemical reactions, and whose cartesian flavour of logical priority is almost certainly fictitious. We shall find it useful to illustrate by means of the Gompertz equation the sort of reasoning which may or may not be based upon the analysis of growth equations.

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* This term is preferable to Huxley's *auxano-differentiation* since the process is not necessarily accompanied by growth in the usual sense. It represents a relationship only partly coextensive with that described by mathematicians as a *homeomorphy*.

Neither the sum nor the average of several Gompertz functions is itself a Gompertz function (Winsor 1932) unless *both* the velocity constants are identical throughout. If they are not very different then, as is the case with the logistic curve, the sum or average may approximate quite closely to the original function.

The relationship between two masses each dependent upon Gompertz functions will be parabolic only if the velocity constant k, representing the rate of decline of the specific growth rate, has the same value in both instances. For if

$$\phi(W_1) = A_1 e^{-k_1 t}, \quad \phi(W_2) = A_2 e^{-k_2 t},$$

then

$$\frac{W_1}{W_2}\frac{dW_2}{dW_1} = \frac{A_2}{A_1} e^{t(k_1-k_2)},$$

which integrates to Huxley's (1932) equation $W_2 = bW_1^a$ ($a = A_2/A_1$) when $e^{t(k_1-k_2)} = 1$, which will be the case when $k_1 = k_2$. Lumer (1937) has shown that if k_1 and k_2 are not very different, then the relationship between the masses of the two systems is approximately parabolic over the earlier part of the life cycle. Huxley (1932, pp. 6–7) does in fact require that the rate of decline of the specific growth rates should be the same for all elements of a biological system, in order to deduce this parabolic relationship as a universal expression for differential growth. The evidence for this assumption, upon which the validity of his reasoning partly depends, is not given.

It is necessary to add that in the Gompertz, as in other growth equations, the point of inflexion has no biological significance whatsoever (Weymouth *et al.* 1931).

The *theoretical basis* of the Gompertz function, if it needs to have one, is simply that the specific growth rate of certain biological systems declines in the exponential fashion which is known to describe the decline of other behaviour characteristics (Brody 1928). It was the plausibility of this assumption that led Sewall Wright (1926) to suggest the use of it in the first place. As a further development, we might adopt Minot's conception that it is the magnitude of the histological differentiation of cells which determines, or is a proper expression of, the magnitude of their specific growth rate. If this is the case, the magnitude (D) of differentiation will depend upon the following function of time:

$$D = a + b(1 - e^{-kt}),$$

in which $a = D$ ($t = 0$) and b is the multiple of a constant converting the specific growth rate into units appropriate to measure the magnitude of differentiation. In the absence of any units of the sort, other than those of Murray's (1926) 'subjective' scale, it might be profitable to measure the magnitude of differentiation in this way by direct reference to the specific growth rate. The equation embodies the plausible hypothesis that differentiation starts with a high initial velocity which declines smoothly at a rate proportional to the defect of the magnitude of differentiation at any instant from its final magnitude. Unfortunately, it is difficult to see in what way a fibroblast *cell* (as opposed to the tissue of which it is an element) is more highly differentiated at 6 months old than it or its linear descendants are at 1 year old; and Minot's theory, according to which differentiation is the physiological basis of senescence, is very difficult to reconcile with the clear demonstration by Hoffman *et al.* that the growth *potencies* of tissues of different ages are identical. *There is in fact nothing irreversible in the consequences of differentiation in so far as they affect the rate of growth.* A good deal of what is commonly called 'senescence' in the modern generalized sense is merely development looked at from the other end; and Minot's epigram, that organisms die fastest when they are young, is often an oblique statement of the fact that they develop more slowly as they grow older. A phenomenon of 'senescence' strictly so called is one which is continuous throughout life with something that contributes directly to the death of the organism. There is no known reason why an animal should die merely because its specific growth rate declines to zero.

The disability which complicates all attempts to propose a relationship between growth and differentiation is that there is no method of measuring the latter numerically, although there are analytical methods of a general nature which are capable of assimilating apparently very diverse forms. In simple instances, it proves to be possible to make comprehensive statements about growth *and* form; this will be explained in another paper.

We have not considered at any point whether the sort of dependence of size on age expressed by a very simple algebraic equation is *a priori* likely or not. Nor is a consideration of the problem likely to be of much value, since there are no standards to which questions of '*a priori* likelihood' can be referred. Only the empirical facts can give one any ground for passing judgement on the appropriateness of the use of mathematical methods in the analysis of growth.

I should like to thank Prof. E. S. Goodrich and Mr J. Z. Young for reading through this paper and offering me their advice; and Mr E. C. Reeve for his assistance in applying certain statistical tests.

SUMMARY

1. A property referred to as the *growth energy* of the chicken's heart, and representing the resistance of freshly explanted tissues to inhibition of their growth, is described and measured.

2. The growth energy is found to decline exponentially over the range 6–18 days.

3. It is shown that the growth energy is proportional to, and therefore a measure of the specific growth rate of, the heart *in vivo*.

4. The growth in mass of the chicken's heart can therefore be described by a mean curve which is that of a Gompertz equation.

5. The growth energy of tissues rises sharply during cultivation *in vitro*, and initial embryonic age differences are smoothed out.

6. The relation between growth, growth energy, cell movement and metabolism is discussed, and a possible system of functional dependence among them considered.

7. The relevance of the Gompertz equation to problems of relative growth and senescence is briefly considered.

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The actions of eserine-like compounds upon frog's nerve-muscle preparations, and conditions in which a single shock can evoke an augmented muscular response

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The muscular responses to one shock, or two shocks separated by 3–10 msec., applied to the nerve of a frog's nerve-sartorius preparation, when immersed in Ringer's solution containing glucose and buffered with bicarbonate, were reasonably constant for about 3 hr. The main response to two shocks was followed by a small after-tension, which, like Bremer's (1932) 'neuromuscular contracture', was abolished by atropine in concentration too small to influence the main response, or by curarine in concentrations which somewhat reduced the main response.

Treating nerve-sartorius preparations from frogs which had been kept at 14–18° C for some days before use ('warm' frogs) with prostigmine, eserine, or the dimethylcarbamic ester of 8-hydroxymethylquinolinium methylsulphate, usually produced no change in the main response to one or to two shocks, but augmented the after-tension in the response to two shocks, and caused an after-tension to follow the response to a single shock.

On the other hand, treating preparations from frogs which had been kept at 0–5° C for more than 40 hr. before use ('cold' frogs) with any of the eserine-like compounds caused the response to one or to two shocks to become augmented, as with mammalian preparations, and to be followed by irregular twitchings which lasted for about a second. The after-twitchings were abolished by atropine in concentrations which slightly reduced the main response, or by curarine in concentrations which reduced the main response rather more.

Treating 'warm' preparations with Ringer's solution of half the usual calcium content, or with Ringer's solution containing any of the following agents (which are known to sensitize to stimuli of long duration), in small concentration, produced no appreciable change in the responses: tetraethylammonium iodide, sodium citrate, guanidine carbonate, or methylguanidine hydrochloride. If, however, preparations so treated were also treated with any of the eserine-like compounds, their responses then became like those of a 'cold' preparation which had been treated with an eserine-like compound.

The responses evoked by applying one shock, or two with an interval

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of 3–10 msec. between them, to the pelvic end of the frog's sartorius muscle, after sufficient curarine to render nerve stimulation ineffective, were uninfluenced by any of the eserine-like compounds, or by any of the five agents mentioned in the previous paragraph, provided that shocks of short duration were used: when condenser discharges of long duration were used, treatment of 'warm' preparations with any of the five agents, in the same concentrations as produced the changes described in the previous paragraph, augmented the response.

Calcium-deficiency, in large or small degree, affected little the response to stimulation of muscle. But a moderate deficiency changed the responses to repetitive stimulation of nerve in such a way as might have been imitated by stimulating the nerve of the untreated preparation at a lower frequency. When the deficiency was more severe, on stimulating with 150 shocks per sec., no response was visible for the first 1–3 sec., the tension then rose to less than a quarter of its normal maximum, and afterwards fell slowly. Prostigmine in large concentrations produced little improvement, whereas calcium quickly produced a marked improvement.

Wedensky found (1883, 1885, 1886) that when the nerve of a frog's nerve-muscle preparation was stimulated maximally for 10–20 sec. at a rate of about 250 shocks per sec. the muscle gave a twitch-like response, and then relaxed almost completely and remained relaxed; that when the nerve was stimulated at a rate of about 50 shocks per sec. the muscle gave a response which rose to a greater maximum than that exhibited during high-frequency stimulation, and then diminished slowly. He showed (1886, 1891*a*, *b*, *c*) that the rapid relaxation was due mainly to a failure of neuromuscular transmission. Recently I have described experiments with frog's nerve-muscle preparations, showing that one of the actions of eserine, prostigmine (Roche), or other eserine-like compounds, was to lower the frequency at which maximal repetitive stimulation of the nerve resulted in failure of muscular response (1938). I showed that in these conditions also the failure of muscular response was due mainly to failure of neuromuscular transmission. Keith Lucas (1911) and Adrian (1913) developed methods of studying neuromuscular transmission in which only two or three shocks need be applied in rapid succession to the nerve. My next aim was, therefore, to see whether more precise information could be obtained using those methods instead of repetitive stimulation. Before I could begin such experiments, however, it was necessary to examine the actions of eserine-like compounds (hereafter this expression includes eserine and prostigmine) upon the response to the more or less synchronous nerve volley set up by an isolated shock. This was all the more necessary because my previous experiments had suggested that in certain conditions eserine-like compounds caused the response of the muscle to a single nerve volley to become repetitive. Some of those experiments were made with preparations from frogs which had been kept at 14–18° C for some days before use ('warm' frogs), others with preparations from frogs which had been kept at 0–5° C for more than 40 hr. before use ('cold' frogs). I found, on the one hand, that the warm frog preparations, after treatment with sufficient eserine or prostigmine, generally gave, at the beginning of stimulation of the nerve with 50–200 shocks per sec., a twitch-like muscular response of maximum tension equal to that which had been elicited by a single maximal shock to the nerve of the untreated preparation; that, on the other hand, cold frog preparations, after the same treatment, gave, at the beginning of stimulation in the same way, a muscular response in which the maximum tension was of such a size as would have been elicited by a train of several shocks to the nerve of the untreated preparation. Moreover, with the cold preparations eserine or prostigmine, in small concentration, augmented the early part of the response to stimulation of the nerve with 20–40 shocks per sec. By experiments described in § 1 of this paper I obtained evidence that eserine-like compounds caused the muscles of cold preparations to respond repetitively to a single nerve volley, but not the muscles of warm preparations. These results led me to examine the conditions in which a frog's nerve-muscle preparation could be made to give an augmented response to a single nerve volley when treated with an eserine-like compound. That examination is described in § 2. Those experiments, in their turn, led me to examine the actions of calcium-deficient solutions together with prostigmine upon the responses of frog's nerve-muscle preparations (§ 3). Part of this work has been described in preliminary communications (1936*a*, *b*). The experiments by Lucas's and by Adrian's methods will be described in the next paper.

MATERIALS

For reasons already discussed, isolated nerve-sartorius preparations were used immersed in glucose-containing Ringer's solution heavily buffered with bicarbonate (Cowan 1938). The frogs—warm or cold—were English *Rana temporaria* which weighed 20–60 g. and which had been in captivity for less than a month. The dissection was made at room temperature. Except where otherwise stated, the preparations from warm frogs were soaked for an hour in Ringer's solution before use, and those from cold frogs for only half an hour. The Ringer's solution was prepared by adding 5 c.c. of a distilled-water solution containing 4 g. $CaCl_2$ per 100 c.c. to 995 c.c. of a distilled-water solution containing 6·0 g. NaCl, 1·34 g. $NaHCO_3$, 0·10 g. KCl, and 1·0 g. glucose. The pH of this solution, when in equilibrium with oxygen-5 % carbon dioxide mixture, at 15° C, was 7·2. When a Ringer's solution containing more or less calcium than that above was required, the volume of the added calcium chloride solution was adjusted accordingly.

I am indebted to Roche Products Ltd., London, and F. Hoffmann-La Roche, Ltd., Basle, for supplies of the following eserine-like compounds: prostigmine, the dimethylcarbamic ester of 8-hydroxymethylquinolinium methylsulphate (hereafter substance 38), and the dimethylcarbamic ester of 3-hydroxyphenylmethyldiethylammonium iodide (hereafter preparation 3393). The last-mentioned compound was specially synthesized for experiments reported in this and in an earlier paper. Solutions of these compounds or of eserine or atropine sulphate were made up freshly in Ringer's solution before each experiment and then diluted as required.

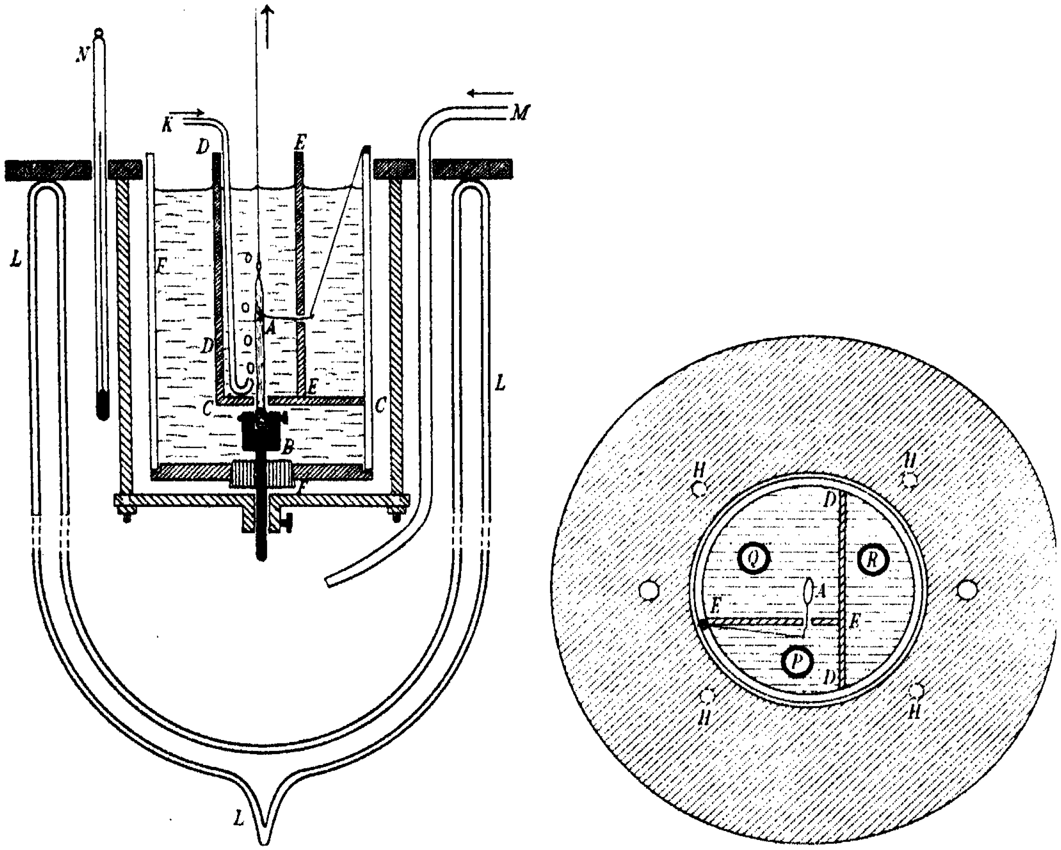
Two samples of purified curarine chloride were used. They were parts of the preparations which Professor C. Lovatt Evans and Dr H. King had been kind enough to give to Dr Ing and myself, and which have been used in previous work (Cowan and Ing 1934, 1935; Cowan 1938). The molecular weight of curarine chloride was assumed to be about 333, from Boehm's (1897) analysis.

For the experiments described in § 2 the sodium citrate was an "AnalaR" sample, the tetraethylammonium iodide, guanidine carbonate, and methylguanidine hydrochloride were prepared by recrystallization.

SECTION 1

METHODS

After the preliminary soaking the preparation was mounted in a bath filled with Ringer's solution which was maintained at 15° C and stirred with oxygen-5 % carbon dioxide mixture. For some experiments the bath was a modified Lucas's trough divided by partitions so as to form fluid electrodes through which the nerve or the pelvic end of the muscle could be stimulated. The stimulating currents were led in through zinc-zinc sulphate half-cells. The muscle was connected to the Lucas's lever crank and thence to an isometric spring torsion lever recording on smoked paper: the moving system had a period of approximately $\frac{1}{30}$ sec. Further details of the foregoing apparatus can be obtained from an earlier description (1938). For other experiments it was convenient to use a new bath which could be immersed in a water thermostat in a Dewar vessel. Details are given in figure 1 and in the description of that figure. The bath was designed primarily for the experiments to be described in the next paper. During



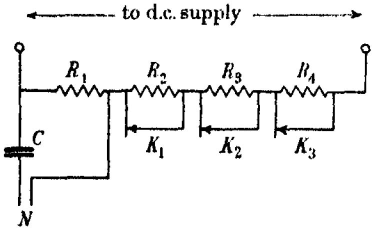
(i) (ii)

FIGURE 1. Vertical type bath, in water thermostat. (i) Section, (ii) Plan. *A*, nerve-sartorius preparation in position. *B*, pin and holder for fixing the acetabulum. *C*, *C*, bakelite partition with shaped hole through which the muscle passes. *DD*, vertical partition. *EE*, a second vertical partition, with hole through which the central end of the nerve passes. For convenience in drawing this partition has been turned through 90° in (i). *FF*, the muscle bath, which was a cylindrical glass sleeve closed at its lower end by a bakelite disk. *HHHH*, the muscle bath was fixed in a cage formed by vertical brass rods with their upper ends screwed into a bakelite plate and their lower ends secured to a thick brass plate. The holder for the acetabulum was clamped in a collar in the centre of the brass plate. The bakelite plate at the top of the cage was held in position by a steel ring, which is not shown here. The tendon of the muscle was tied to a steel wire, the other end of which was connected to an isometric lever. *K*, jet conveying oxygen-5 % carbon dioxide mixture. Although not shown, similar jets were provided in the other compartments of the bath. By this means the solution was kept stirred and at the correct pH. *LLL*, water-filled Dewar vessel. *M*, air jet for stirring the water. *N*, thermometer. *P*, *Q*, and *R*, zinc-zinc sulphate half-cells for leading stimulating current into the compartments. For stimulation of nerve *P* and *Q* were used; for stimulation of muscle *Q* and *R*.

those experiments I wished to maintain the temperature of the bath and its contents constant to within about ±0·1° C. With the earlier bath, maintenance of the temperature within these limits would have needed too much attention.

For the stimulation of nerve or of muscle break induction shocks or condenser discharges were used. The break shocks were obtained from a coreless coil with a Lucas's pendulum in the primary circuit. The position of the secondary coil relative to the primary was kept fixed and the strength of shock adjusted by altering the current in the primary. The duration of the break shock was not measured, but Mr A. L. Hodgkin (Hodgkin 1937) found that in his experiments with another coil of the same type the duration of a shock was about 40 μsec. In my experiments the external resistance in the secondary circuit was probably lower owing to the use of fluid electrodes, and for this reason probably the duration was a little longer. When condenser discharges were used, the condensers were of known capacities and the effective shunting resistance was measured with alternating current: the durations (time-constants) of the discharges were calculated with these figures. The strength of stimulation was altered by altering the voltage to which the condenser was charged.

For experiments in which two shocks were required either break shocks or condenser shocks were used. The interval between the shocks was timed by a Lucas's pendulum. The break shocks were obtained from two coreless coils with their windings placed at right angles to one another, and with their secondary windings connected to the same electrodes. The positions of the secondaries were kept fixed relative to the primaries, and the strengths of the shocks, always with both secondaries in circuit, were adjusted by altering the currents in the primaries. The condenser shocks were either two discharges in succession or else a discharge followed by a charge. The two discharges were obtained by a simplification of the arrangement shown in figure 2A—the key K_3 was short-circuited during these experiments. The discharge shock followed by charge was obtained by the arrangement shown in figure 2B. At first, I attempted to use the electric supply mains, 110 V D.C., as a source of current. Since, however, they had a superimposed A.C. ripple sufficiently intense to stimulate nerve, I had to abandon them in favour of a 120 V battery of accumulators. The strengths of the two shocks could be varied relative to one another by adjusting the values of the resistances R_1, R_2, R_3 in figure 2A, or R_1, R_2, R_3, R_4 in figure 2B: the strengths of the shocks could also be varied simultaneously by tapping off different voltages from the battery. The sum of the resistances $R_1+R_2+R_3$ in figure 2A, or $R_1+R_2+R_3+R_4$ in figure 2B, was kept as small as possible in order that the resistance of the battery arm of the circuit should be small in comparison with the resistance of the arm which included the fluid electrodes and tissue: at the same time, however, R_1 in figure 2A, or R_1+R_3 in figure 2B, could not be reduced below certain minimum values, because otherwise, when K_1 and K_2 were closed, the current drawn from the battery would have been excessive. The time-constant of the shocks was altered by altering the capacity of the condenser, C. It is to be noted that with the arrangement shown in figure 2B the duration of the charge shock was longer than that of the discharge, but it was possible by choosing suitable values for the resistances R_1, R_2, R_3, R_4 to see that the prolongation did not exceed 20 % at worst (see the example in the description of figure 2B).



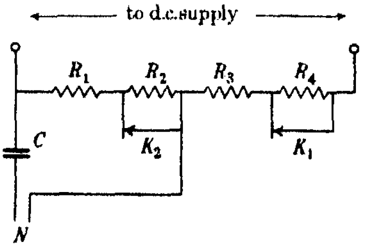


FIGURE 2 (left). Circuit for obtaining two or three condenser discharges in rapid succession. R_1, R_2, R_3, R_4, resistances. K_1, K_2, K_3, keys of a Lucas's pendulum. C, condenser. N, fluid electrodes leading current to muscle or nerve.

FIGURE 2 (right). Circuit for obtaining condenser discharge followed by charge. R, K, C, and N, have the same meanings as in figure 2 (left).

In one experiment the resistance between the fluid electrodes was 4600 ohms. The capacity of the condenser was $0{\cdot}02\,\mu$F. It was desired to discharge and then to charge the condenser by 20 V. This was done by applying 80 V across the "D.C. supply" terminals, by making $R_1 : R_2 : R_3 : R_4 = 3 : 6 : 1 : 2$, and by making $R_1 + R_3 = 640$ ohms. The bringing of R_2 into the arm of the circuit which included the fluid electrodes and tissue made the time-constant of the charge shock $960/(4600+480) = 18{\cdot}9$ % longer than that of the discharge shock.

After the preparation had been mounted, the procedure was to record the responses to test shocks applied to the muscle or to the nerve. When the responses had become steady, the substance whose action was to be examined was added in the form of a small volume of concentrated solution to the compartment which contained the greater part of the muscle, or else the bath was drained and then filled with fresh Ringer's solution containing the substance. If, after the action of the first substance on the response had been recorded, the subsequent action of a second substance was to be examined, either the second substance was added in concentrated solution to the 'muscle' compartment, or the solution in the bath was removed and then replaced after addition of the second substance.

RESULTS

'Cold' preparations which had been subjected to a preliminary soaking in Ringer's solution for less than an hour exhibited a few spontaneous twitchings soon after being treated with any of the eserine-like substances. Generally, the twitchings continued for 2–3 min. and then disappeared. In contrast to this, 'cold' preparations which had been soaked for more than an hour rarely exhibited twitchings; 'warm' preparations never exhibited twitchings (see also Cowan 1938).

The responses of preparations from warm frogs to direct stimulation of the muscle

Under this subheading, and under subsequent ones, I shall first describe the action of prostigmine, and afterwards the actions of the other eserine-like substances.

Treatment of nerve-sartorius preparations with prostigmine in concentrations 0·03–3000μM (micromols per litre), for periods up to 8 hr., produced no greater change in the response to a single shock than occurred in the response of control preparations which were merely soaked in Ringer's solution. If before treatment and during the first hour of treatment the test shocks were limited to groups of two or three with 10–30 sec. intervals between the shocks and 5 min. intervals between the groups, and afterwards to similar groups at 10 min. intervals, the maximum tension developed in the response usually declined by less than 10% in 3 hr. In the experiments which were continued for 8 hr. the test shocks were applied in accordance with the foregoing plan before and during the first hour of treatment, then the preparation was left in prostigmine solution and more test shocks—one or two groups—were applied at intervals of $\frac{1}{2}$–1 hr. In about 8 hr. the maximum tension developed in the responses of the prostigmine-treated and of the control preparations declined to 80–95% of what it had been at the beginning of the experiment.

When Ringer-soaked muscles were stimulated by two successive shocks at an interval of 3–10 msec. some gave a summated response followed by a small tension which diminished and disappeared in $\frac{1}{2}$–2 sec., others gave a summated response followed by no perceptible after-tension. Those preparations which gave an after-tension usually did so consistently during an observation period of about 3 hr.; the maximum value of the after-tension rarely exceeded 2–4 % of the tension attained in the main response. The treatment of Ringer-soaked preparations with prostigmine in concentration 0·03–300μM produced no change in the summated response, or in the after-tension when present. The after-tension was unaffected by curarine chloride in concentration 1μM, or by atropine sulphate in concentration 1 in 20,000.

In the foregoing experiments the shocks were applied to the pelvic end of the muscle in such a way that the cathode was always the more central of the electrodes (see Rushton 1932). The results were unaffected by varying the time-constant of the shocks from 40 to 500μsec., or their strengths from 1 to 1·5 times those needed to evoke maximal response.

When tested in the way just described, eserine in concentration 0·03–50μM, or substance 38 in concentration 0·03–30μM, produced no change in the response of muscle to single shocks or to pairs of shocks. Preparation 3393 produced no change in the response of muscle to shocks of time-constant 40–250μsec. If, however, a single shock of time-constant 3000–5000μsec. was used, a (somewhat supramaximal) strength could be found such that whilst the muscle was immersed in Ringer's solution the response was of the same size as was evoked by a short maximal shock, but such that after the muscle had been treated for 10–20 min. in a solution containing 50μM of preparation 3393 the response became augmented by up to about 30 %. Augmentation of the response also occurred after treatment with a solution containing 10μM of preparation 3393, but it was less consistent and smaller.

The responses of preparations from warm frogs to stimulation through the nerve

The treatment of preparations with prostigmine usually produced no change in the maximum tension evoked from the muscle by a maximal shock applied to the nerve, but occasionally it produced a slight increase. Apart from this prostigmine caused a small tension to appear after relaxation was almost complete. The size of the after-tension differed in different preparations, its maximum value ranging from 3 to 15 % of the maximum tension developed in the main response. The after-tension gradually vanished in 1–4 sec. When prostigmine was used in a concentration 0·03μM the change did not become very well developed, but when higher concentrations were used the change became well developed in times about the same or a little longer than those required for prostigmine, in the same concentration, to exert its full effects on the response to repetitive stimulation through the nerve: with 0·3μM solution the time required was about 1–1½ hr., with 300–3000μM solution it was 5–10 min. (cf. Cowan 1938). No changes other than the foregoing ones occurred so long as prostigmine-treated preparations were not stimulated frequently. If, however, preparations which had been treated with a solution containing 300–3000μM were stimulated at regular intervals of 15–60 sec., according to the condition and sensitivity of the preparation, then after one or two responses the maximum tension suffered a temporary depression which was followed by a slow recovery during later responses. The depression and recovery have already been described in some detail (Cowan 1938).

Two shocks with an interval of 3–10 msec. between them elicited from Ringer-soaked preparations a summated response followed immediately by an after-tension which declined and vanished in 1–2 sec. Usually the maximum value of the after-tension amounted to 3–10 % of the tension attained in the main response. When the interval between the shocks exceeded 10 msec., little or no after-tension could be observed. One of the actions of prostigmine was to prolong the minimum interval at which two shocks could elicit a summated muscular response (see next paper). With prostigmine in concentrations 0·03–6·0μM, it was possible, however, to make the interval between the shocks great enough to eliminate this action, and yet not so great that the after-tension was absent from the responses before treatment. If the shocks were so spaced, the maximum tension of the summated muscular response was unaffected by the prostigmine, whereas the after-tension was considerably augmented in size and duration (figure 3). Moreover, this after-tension following the summated response to two shocks was greater than that which the prostigmine caused to appear following the response to a single shock.

The after-tension, whether evoked by two shocks to the nerve of a Ringer-soaked preparation, by one or by two shocks to the nerve of a prostigmine-treated preparation, could be reduced or abolished by curarine, or by atropine, in small concentration. Generally the concentration of curarine chloride which abolished the after-tension was about 0·1–0·5μM, one which also reduced the tension developed in the main response. The concentration of atropine sulphate which abolished the after-tension was from 1 in 100,000 to 4 in 100,000; a perceptible reduction of the main response usually did not occur unless the concentration was raised to 1 in 10,000.

The responses described in the three preceding paragraphs were uninfluenced by alterations in the sense of the shocks (cathode proximal or distal)—either single shocks or two in rapid succession. The responses were also uninfluenced by varying the time-constants of the shocks between 40 and 250μsec., or their strengths between 1 and 1·5 times those needed to evoke maximal response. The responses described in the subsections of this paper beginning on pp. 367, 377 and 383 were likewise independent of the characteristics of the shocks, between the same limits.

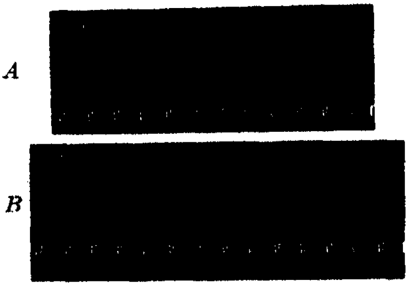


FIGURE 3. 26. iv. 36, Experiment 1. The effect of prostigmine upon the muscular response to stimulation of the nerve of a sartorius nerve-muscle preparation which had been taken from a warm frog. The stimulus consisted of two shocks, each of time-constant approximately 40 μsec., with an interval of 8·0 msec. between them. The time markings are seconds.

A, 12.6 p.m., the Ringer-soaked preparation was stimulated. 12.10 p.m., the bath was drained and then filled with Ringer's solution containing 6μM prostigmine.

B, 1.16 p.m., the preparation was stimulated as before.

The actions of eserine and of substance 38 were qualitatively very similar to those of prostigmine. No fully quantitative comparison of the activities of these compounds was attempted, but the results of the various experiments, when taken together, probably justify a rough estimate that eserine in concentration 0·15–50μM was equivalent to prostigmine in concentration 0·03–15μM, and that substance 38 in concentration 0·03–30μM was equivalent to prostigmine in the same concentration. The actions of preparation 3393 were qualitatively different from those of the other substances. This substance caused the responses to one or two shocks to become like those of cold frog preparations which had been treated with prostigmine (see below).

The responses of preparations from cold frogs to direct stimulation of the muscle

Prostigmine, eserine, substance 38, or preparation 3393, in the same concentrations as in the experiments with warm muscles, produced no change in the response to one or two maximal or slightly supramaximal shocks of time-constant 40–500μsec. But when shocks of longer duration (time-constant 3000–5000 μsec.) were used, the response changed as a result of merely soaking the preparation in Ringer's solution. If the soaking was only for about half an hour, generally, I was unable to find a range of stimulation strengths over which the size of the response remained constant, i.e. a response which could be clearly identified as maximal; instead the size of the response seemed to vary with the strength of stimulus. After the preparation had been soaked for about a further 2 hr., however, it became possible to find a small range of stimulus strength over which the size of the response remained constant and approximately equal to (or sometimes a little greater than) that of the response to a shock of short duration. On examining again the responses that the preparation had given after soaking for only half an hour in Ringer's solution, it could be seen that a shock of long duration, and of strength which was found to be maximal after about 2½ hr. soaking, had evoked an augmented response. Prostigmine, eserine, or substance 38 produced no change in the response to shocks of long duration. Preparation 3393 when used in concentration 50 μM produced an augmentation which outweighed the effect of soaking in Ringer's solution, but when used in concentration 5 μM this did not always happen.

The responses of preparations from cold frogs to stimulation through the nerve

The responses of Ringer-soaked preparations to one or two maximal shocks did not differ in any obvious way from the corresponding responses of warm preparations. When cold preparations which had been soaked in Ringer's solution for ½–1 hr. were treated with prostigmine in concentration 0·03–0·3 μM, the response to a single shock became augmented. The response to two shocks separated by an interval of 3–10 msec. not only became augmented but was followed immediately by irregular twitchings. Usually the twitchings lasted for about a second. Figure 4*A–D* shows responses of the foregoing types. (It is immaterial that in this instance prostigmine and an agency other than cold-conditioning of the frogs before use were responsible for the changes. See later.)

When cold preparations which had been soaked in Ringer's solution for ½–1 hr. were treated with prostigmine in larger concentrations (3–10 μM), the response evoked by a single maximal shock became augmented and was followed immediately by irregular twitchings which lasted for about a second and then merged into a smooth after-tension. Figure 5*A–C* shows changes of this kind. Similarly, when two shocks were used, with an interval of 3–10 msec. between them, prostigmine augmented the response and caused irregular twitchings which later merged into an after-tension to follow it. The tension developed in the augmented response to two shocks was greater than that developed in the augmented response to one shock; moreover, the twitchings which followed two shocks were of greater size and duration than those which followed one shock. Differences of this kind are shown in figures 5*C* and 6. In many experiments, about 10 sec. after the twitchings and after-tension which followed one or two shocks had subsided, a few fresh twitchings appeared (e.g. in figure 6). Although these were less vigorous than the earlier twitchings, sometimes they continued for nearly a second. If stimulation was repeated more frequently than once in 2 or 3 min., the late twitchings became still later or dis-

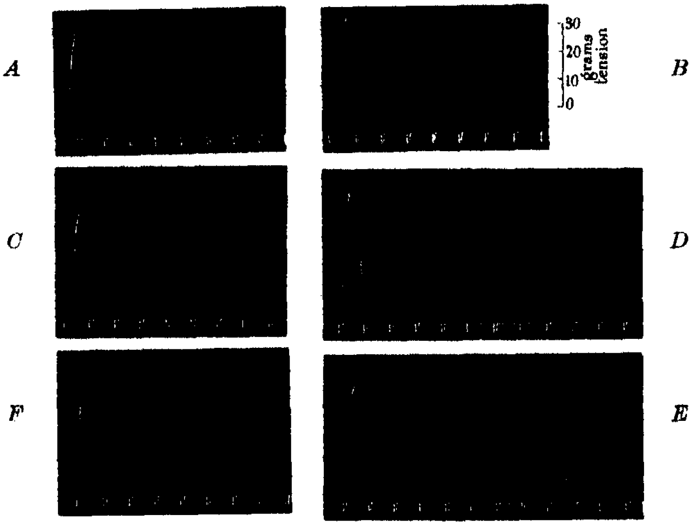


FIGURE 4. 27. iv. 36, Experiment 2. The effect of prostigmine, in small concentration, upon the response to stimulation of the nerve of a sartorius nerve-muscle preparation which had been taken from a warm frog and subjected to a preliminary soaking in Ringer's solution of one-half the usual calcium chloride content: the effect of atropine subsequently. The time markings are seconds.

3.50 p.m., preparation immersed in the modified Ringer's solution. *A*, 4.25 p.m., nerve stimulated with a single shock of time-constant approximately 40 μsec. *B*, 4.32 p.m., nerve stimulated with two shocks, each of time-constant approximately 40 μsec., at an interval of 3·9 msec.

5.4 p.m., bath drained and then filled with modified Ringer's solution containing 0·3μM prostigmine. *C*, 5.30 p.m., nerve stimulated with a single shock as in *A*. *D*, 5.58 p.m., nerve stimulated with two shocks as in *B*.

5.59 p.m., atropine sulphate added to solution in bath to make the concentration 1 in 25,000. *E*, 6.8 p.m., nerve stimulated with two shocks as in *B*. *F*, 6.25 p.m., nerve stimulated with a single shock as in *A*.

appeared temporarily from the response. The early twitchings were also sensitive to frequent stimulation, although different preparations differed

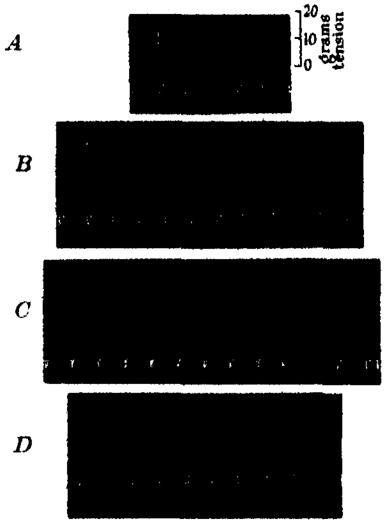


FIGURE 5. 26. iv. 36, Experiment 2. The effect of prostigmine, in larger concentration, upon the response to stimulation of the nerve of a nerve-sartorius preparation which had been taken from a warm frog and subjected to a preliminary soaking in Ringer's solution containing one-half the usual amount of calcium chloride: the effect of curarine subsequently. The time markings are seconds.

4.10 p.m., preparation immersed in the modified Ringer's solution. *A*, 4.55 p.m., nerve stimulated with a single shock of time-constant approximately 50 μsec.

5.5 p.m., bath drained and then filled with modified Ringer's solution containing 6 μM prostigmine. *B*, 5.36 p.m., nerve stimulated with a single shock as in *A*. *C*, 6.10 p.m., nerve stimulated with a single shock as in *A*.

6.11 p.m., curarine chloride added to bath to make the concentration 0·5 μM. *D*, 6.23 p.m., nerve stimulated with a single shock as in *A*.

FIGURE 6. 26. iv. 36, Experiment 2. Part of the same experiment as in figure 5.

6.3 p.m., nerve stimulated with two shocks, each of time-constant approximately 50 μsec., with an interval of 4·0 msec. between them.

a good deal in this respect. Generally, in a second response about half a minute after a first response the twitchings were much reduced, and were fused together more than in the first response. In a third response about half a minute after a second response the twitchings became still more

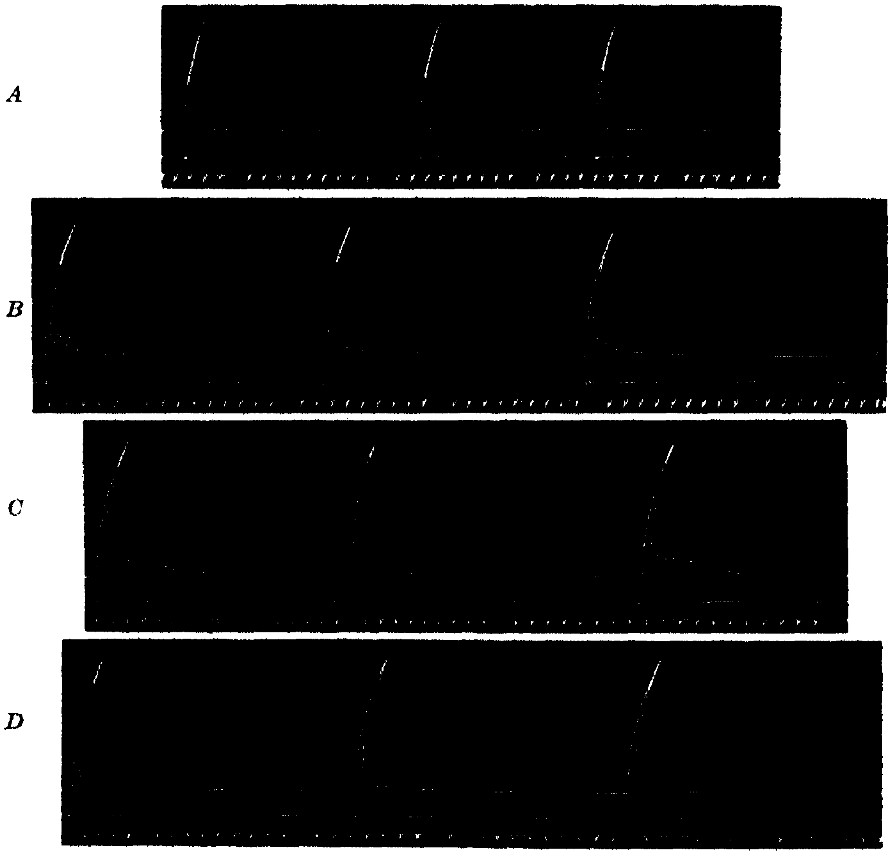


Figure 7. 15. vi. 38. The effect of prostigmine upon the response to stimulation of the nerve of a Ringer-soaked sartorius preparation from a warm frog (Hungarian *R. esculenta*): the effect of guanidine added later: the effect of atropine added still later. The time markings are seconds.

12.30 p.m., preparation immersed in Ringer's solution.

1.16 p.m., bath drained and then filled with Ringer's solution containing 8μM prostigmine. *A*, 2.23 p.m., series of three shocks to the nerve, each with a time-constant of approximately 160 μsec.

3.1 p.m., guanidine carbonate added to bath to make the concentration 420μM (i.e. 840 micro-equivalents per litre). *B*, 3.55 p.m., nerve stimulated three times as in *A*.

4.26 p.m., more guanidine carbonate added to bath, making the total concentration 1840μM (3680 micro-equivalents per litre). *C*, 4.54 p.m., nerve stimulated three times as in *A*.

4.58 p.m., atropine sulphate added to bath to make concentration 1 in 100,000. *D*, 5.9 p.m., nerve stimulated three times as in *A*.

fused, and the tension developed was larger than the tension developed in the twitchings following the second response. The augmented main response was relatively less sensitive to frequent stimulation; it did not diminish during stimulation three times per minute. Figure 7*B* illustrates the effects of frequent stimulation, although in all those responses the twitchings are smaller relative to the main response, and more fused together than was usual with preparations from *R. temporaria* (see further note in § 2).

The responses described in the foregoing paragraph became fully developed within about an hour of subjecting the preparation to Ringer's solution containing prostigmine. If the soaking in that solution was then continued for another 1–2 hr., the time depending on how sensitive the preparation was and how long it had previously been soaked in Ringer's solution, further slow changes occurred: first, the late twitchings following one or two shocks disappeared; later, the maximum tension developed in the augmented main response became slightly smaller, and the early twitchings became smaller and more fused together; still later, a single shock evoked an augmented response followed by a smooth after-tension, whereas two shocks, in rapid succession, evoked an augmented response followed by twitchings which later merged into a smooth after-tension.

Prostigmine in concentrations 10–100μM reduced the time required for development of the augmented responses and twitchings described above. It produced no further change, except perhaps that it made the preparations rather more sensitive to the effects of frequent stimulation.

The actions of curarine and of atropine upon the responses of prostigmine-treated preparations were examined. Curarine chloride in concentration 0·1–0·5μM was required to abolish the early and the late twitchings following one or two shocks. The curarine chloride also reduced the augmented main response, although generally not so much that the augmentation was completely nullified (figure 5*C*, *D*). Curarine chloride in smaller concentrations (0·01–0·05μM) reduced the early twitchings and abolished the late ones if they were present, but it did not affect the main response. Atropine sulphate in concentration 1 in 100,000 to 4 in 100,000 abolished the early and late twitchings, and in most experiments, reduced the augmented main response (e.g. figure 4*D*, *E*): usually the reduction was less than 10%. Atropine sulphate, in concentration 1 in 10,000, reduced the main response below its pre-augmentation value.

The results of experiments with eserine or substance 38 were very similar to those with prostigmine. The activities of these compounds were compared using an approximately quantitative method like that used previously to compare the activities of the same compounds upon the response to repetitive stimulation (Cowan 1938). Eserine sulphate in concentration 0·15–30μM, or substance 38 in concentration 0·03–10μM, was approximately equivalent to prostigmine in concentration 0·03–10μM. Preparation 3393 in concentrations 1–10μM also produced changes similar to those which prostigmine produced. When smaller concentrations were used (0·03–0·06μM) the responses of preparations which had been treated with preparation 3393 differed from those of preparations which had been treated with prostigmine in that irregular twitchings sometimes followed the augmented main response to a single shock.

Discussion

That a shock of long duration applied to the pelvic end of a cold muscle should elicit an augmented response whereas a shock of short duration elicited a response of about the same size as (or a little smaller than) that elicited by a maximal shock to the nerve accords with already established observations. It is known that muscle or nerve from cold frogs responds repetitively to electric currents of long duration whereas muscle or nerve from warm frogs may yield but one response (see e.g. Biedermann 1896, 1898; Katz 1936). The interesting point is that eserine-like substances caused the response of cold muscle to stimulation through its nerve to become augmented. Since the response of the muscle to direct stimulation was unaffected by the eserine-like substances (except preparation 3393 which will be discussed later), since the response evoked by a single shock to the nerve was independent of the character of that shock so long as its strength was at least maximal, and since two shocks with an interval of about 3 msec. between them—an interval less than twice the absolute refractory period of the most rapid motor nerve fibres supplying the muscle (Adrian 1913; see also § 2 of next paper)—elicited a greater response than did one shock, it is very difficult to escape the conclusion that the eserine-like substances caused the muscle to respond repetitively to a single nerve volley, i.e. that they caused the stimulus delivered through the nerve ending to persist at a supraliminal value for more than one refractory period of the muscle. The eserine-like compounds might lower the threshold of the part of the muscle fibre juxtaposed to the nerve ending, and perhaps of the remainder of the fibre also. They might intensify or prolong the stimulus delivered through the nerve ending. They might act by a combination of these methods. If this be accepted, then the observation that eserine-like substances did not cause the response of warm muscle to a single nerve volley to become repetitive can be attributed to the known insensitivity of warm muscle to stimuli of long duration. There are two possible explanations of the slight augmentation of response that sometimes occurred. It may have been due to repetitive response of a few of the fibres of the muscle, or to the bringing into action of a few additional fibres of the muscle. Adrian and Lucas (1912) found evidence that in certain not very well-defined conditions an isolated nerve volley failed to excite some of the fibres of frog muscle whereas a second volley following 20–40 msec. after an earlier one was effective (see also Samojloff 1908).

The above explanation of the augmented responses of cold preparations is, I think, supported by considering the actions of preparation 3393 upon warm preparations. The experiments in which the muscle was stimulated directly indicate that preparation 3393, a derivative of the phenylmethyldiethylammonium cation, sensitizes to stimuli of long duration. This sensitizing action is in accord with what is already known about the properties of cations of similar chemical constitution. Tetraethylammonium cations produce spontaneous activity of nerve (Loeb and Ewald 1916) and of frog's nerve-muscle preparations (Marshall 1914–16). Marshall has shown that the ability to produce spontaneous activity declines and eventually disappears as the ethyl groups of the tetraethylammonium cation are replaced successfully by methyl groups. Walter and I (1937) have shown that tetraethylammonium cations bring about spontaneous activity of nerve as a limiting result of sensitizing it to stimuli of long duration. Turning again to the augmented response of the muscle when stimulated through its nerve, the question might be asked: Does not preparation 3393 produce the augmentation by causing the nerve to respond repetitively? Certainly there is no reason to suppose that the nerve escaped being sensitized to stimuli of long duration, for it is known that other agencies which sensitize muscle also sensitize nerve, e.g. calcium deficient Ringer's solution sensitizes muscle (Loeb 1905; Lucas 1908; Mines 1908*a*, *b*) and nerve (Kahn 1911; Solandt 1936). The answer is that, as in the experiments with "cold" preparations, only short shocks of not greatly supramaximal strength were used in order to avoid repetitive response of the nerve. Since the response to two such shocks separated by an interval of 3 msec. was greater than the response to one shock, it seems that this precaution was effective. Besides a sensitizing action to stimuli of long duration, preparation 3393 has an eserine-like activity on neuromuscular transmission—measured by the changes which it produces in the response of frog's nerve-muscle preparations to repetitive stimulation

(Cowan 1938)—approximately equal to that of prostigmine. Apparently a single nerve volley cannot evoke a repetitive response from the muscle of a warm frog preparation which has been treated with an eserine-like substance, unless that muscle has also been sensitized to stimuli of long duration.

The properties of the after-tension which followed the main response of Ringer-soaked warm muscle to direct stimulation are similar to those of the contracture which Bremer (1932) observed, following the response of the frog's blood-circulated gastrocnemius to direct stimulation by two shocks in rapid succession. In most of my experiments in which the after-tension appeared, probably it was smaller than the contracture in Bremer's, but I cannot be certain of this since his responses were recorded isotonically and mine isometrically. So also, the properties of the after-tension in the response evoked from Ringer-soaked warm preparations by applying two shocks to the nerve are very similar to those of the neuromuscular contracture which Bremer found in the responses of blood-circulated sciatic-gastrocnemius preparations, although in my experiments the after-tension was usually smaller than that in Bremer's published records of isometric responses. The smallness may be due to a difference between the gastrocnemius and sartorius, or to differences of experimental method. Further discussion here would be premature.

For the purpose of the present discussion the most important differences between the two after-tensions described above are: that the one evoked by indirect stimulation was augmented by the action of the eserine-like substances, whereas the one evoked by direct stimulation was not so augmented; that after augmentation, the one evoked by indirect stimulation retained its similarity to Bremer's neuromuscular contracture inasmuch as, compared with the main response, it remained relatively sensitive to atropine and relatively insensitive to curarine.

Apparently the early twitchings in the response of cold preparations which had been treated with an eserine-like substance correspond to the augmented after-tension in the response of warm preparations which had been treated with an eserine-like substance. Bremer (1932, p. 80) has suggested that the contracture is 'the reactional expression of a very slow, specific excitability of the muscle fibre'. If, however, his view is accepted, it is difficult to explain why the after-tension should be partly transformed into twitchings. It seems to me preferable to consider the 'neuromuscular contracture' in the light of what is known about the stimulation of muscle by electric currents. A constant current too weak to set up propagated excitation may produce a contracture localized at the cathode (Biedermann 1896; Bremer 1932). A current which increases too slowly to set up propagated excitation may produce a similar contracture (Lucas 1907, 1908; Fabre 1927–33). It is unnecessary, however, to make any assumption as to what is the nature of the agent which transfers excitation from motor nerve to voluntary muscle. It is sufficient to suppose that the after-tension is due to a stimulus-remainder which persists near the nerve endings for a time long in comparison with the refractory period of muscle, or to an after-discharge at the nerve endings. The augmentation of the after-tension by eserine-like compounds would be consistent with their exerting any of the actions mentioned above when discussing the main responses to a nerve volley. Nor then would it be surprising that through such an action of an eserine-like compound, combined with a sensitization of the muscle to stimuli of long duration, the stimulus-remainder, or after-discharge, should become able to set up propagated excitation in some of the muscle fibres.

SECTION 2

Experiments were made in order to test the above reasoning. If the augmented responses of cold preparations which had been treated with an eserine-like substance, or of warm preparations which had been treated with preparation 3393, to stimulation through the nerve, were due to the muscle being sensitive to stimuli of long duration, then it should be possible to obtain similar augmented responses from warm preparations by treating them with prostigmine, eserine, or substance 38, together with any agent which sensitizes to stimuli of long duration. It was clear that, if the experiments were to be comparable with the earlier ones, the sensitizer would have to augment the responses after the preparations had been treated with prostigmine, eserine, or substance 38, but not before. Any agent sensitizing muscle to stimuli of long duration might be expected, moreover, partly to transform the augmented after-tension into twitchings. Experiments with five sensitizing agents are described below. The experiments were qualitative rather than quantitative, but the five agents were selected with a view to more quantitative experiments which I hope to describe in a subsequent paper. The sensitizing actions of the five agents upon nerve have already been examined in a partly quantitative way: Ringer's solution of low calcium content (Solandt 1936; Katz 1936), sodium citrate (Solandt 1936; Katz 1936), tetraethylammonium iodide (Cowan and Walter 1937), guanidine and methylguanidine (Cowan 1940).

Fühner (1908) has asserted that the spontaneous twitchings of muscle which guanidine produces are due to an action on motor nerve endings. However, Camis (1909) found that guanidine produced spontaneous twitchings of the frog's sartorius muscle after section and degeneration of the nerves. Also, he found that when induction shocks from an iron-cored coil were used to stimulate the sartorius at its pelvic end, guanidine in small concentrations (115–3140μM) caused the response to become augmented; and that guanidine in larger concentrations first augmented and later depressed the response.

RESULTS

Responses to direct stimulation of muscle

The soaking of warm preparations for periods of ½–3 hr. in Ringer's solution of one-half the usual calcium chloride content together with curarine chloride in concentration 5μM, or in Ringer's solution containing any one of the following substances in the concentration stated and curarine in concentration 5μM, produced no change in the response to one shock, or to two shocks with an interval of 3–10 msec. between them:

1000–2000μM, sodium citrate;
20–100μM, tetraethylammonium iodide;
100–500μM, guanidine carbonate;
200–1000μM, methylguanidine hydrochloride.

With the cathode as the more proximal electrode, this result was unaffected by varying the time-constant of the shocks from 40 to 500μsec., and their strengths from 1 to 1·5 times those needed to evoke maximal response. If, however, a single shock of time-constant 3000–5000 μsec. was used, and its strength was so adjusted that the response of Ringer-soaked muscle was of the same size as that evoked by a shock of shorter time-constant, then on treating the muscle with a sensitizing solution usually the response became augmented. The augmentation was reversible. It could be made to disappear by washing the preparation in unmodified Ringer's solution for 20–50 min.

As in the experiments described in § 1, a small after-tension sometimes followed the main response of the preparation before it had been treated with any of the above-mentioned sensitizing solutions. These solutions left the after-tension unchanged or augmented it slightly.

When preparations which had been treated with a sensitizing solution were subsequently treated with prostigmine in concentration 0·03–10μM, eserine in concentration 0·1–50μM, or substance 38 in concentration 0·03–10μM, their responses changed no more in about 3 hr. than did the responses of preparations which were treated only with sensitizing solution.

Responses to stimulation of preparations through the nerve

The treatment of warm preparations for ½–3 hr. with a solution containing any of the sensitizing agents in the concentration specified under the preceding subheading, but no curarine, usually produced no change in the main response to one shock, or to two shocks separated by an interval of 3–10 msec. The after-tension was also unaffected, or slightly augmented. When sensitized preparations were treated with prostigmine in large or in small concentration, their responses became like those of the cold preparations which had been treated with prostigmine in the same concentration. With prostigmine in concentration 0·3–10μM and any of the sensitizers, the resemblance held good even to the appearance of the late twitchings. The sensitizer actually responsible for the augmented response and twitchings shown in figures 4 and 5 was Ringer's solution of one-half the usual calcium content.

The actions of prostigmine in concentrations 0·3–10μM upon the response of any of the sensitized preparations became fully developed in times which were approximately the same as those in which its actions upon the responses of cold preparations became fully developed. If the sensitized preparations were left for about another hour in sensitizing solution which contained prostigmine, little further change occurred in the response. The actions of the sensitizing solution could be reversed, however, by replacing it by unmodified Ringer's solution containing prostigmine in the same concentration. When this was done, within about an hour, the response became almost the one ordinarily given by prostigmine-treated warm preparations.

The changes described above could be produced equally well if the preparation were first treated with prostigmine, and not sensitized until the action of the prostigmine had become fully developed. In these experiments some augmentation of the response to one or two shocks appeared within a few minutes of subjecting the preparation to the sensitizing solution, but the augmentation did not become fully developed for 20–60 min. In some experiments it could be seen also that the after-tension passed through a stage of further augmentation before it burst into twitchings.

A few experiments were made with preparations which had been soaked for 5–6 hr. in Ringer's solution containing prostigmine. Although the muscles had become somewhat oedematous, replacement of the Ringer's solution by a sensitizing solution containing prostigmine in the same concentration caused the response to become augmented and to be followed by twitchings.

The early twitchings, and the late twitchings when present, could be abolished by curarine or by atropine. The effective concentration of curarine chloride ($0{\cdot}1$–$0{\cdot}5\,\mu$M) also reduced the main response by up to 30%. The effective concentration of atropine sulphate (1 in 100,000 to 4 in 100,000) reduced the main response little, if at all.

The changes produced by prostigmine could be produced equally well by eserine or by substance 38.

According to Bremer (1928), neuromuscular contracture, following the main response to two nerve volleys applied at an interval of about 3–10 msec., can be obtained in blood-circulated sciatic-gastrocnemius preparations in the toad and in *R. temporaria*, but not in *R. esculenta*. He has suggested (1932) that the difference is due to the greater ease with which contracture can be evoked in the leg muscles of *R. temporaria*. In view of this difference, I thought it worth while to make a few experiments on the actions of prostigmine and sensitizing agents upon the responses of nerve-sartorius preparations from *R. esculenta*. With warm Ringer-soaked preparations, I was unable to obtain any appreciable after-tension following the response evoked by two shocks to the nerve. Treatment of preparations with prostigmine caused an after-tension to follow the main response evoked by a single shock to the nerve, and a larger after-tension to follow the main response evoked by two shocks with an interval of 5–20 msec. between them. Both of these after-tensions were relatively smaller than in the responses of preparations from *R. temporaria* (cf. figures 3*B* and 7*A*). Treatment of preparations from *R. esculenta* with a sensitizing agent and prostigmine caused the response evoked by a single shock to the nerve to become augmented, and to be followed by early and late twitchings. The early twitchings were relatively smaller and apparently somewhat more fused together than in the responses of preparations from *R. temporaria* (figure 7*B*). I also examined the action of guanidine in higher concentrations upon the responses of preparations from *R. esculenta*. A concentration of 2000–6000$\,\mu$M caused the response evoked by a single shock to the nerve of a Ringer-soaked preparation to become augmented. Treatment of preparations, previously treated with prostigmine, with guanidine in the foregoing concentration did not augment further the main response evoked by a single (short) shock to the nerve, but seemed to cause the early twitchings to become more fused together and to continue for longer, so that they became fused with the late twitchings (e.g. figure 7*C*). Without electrical records, however, it is impossible to be sure how much of the response was due to rapid asynchronous twitching, and how much to contracture. Both the after-tension following the response of unsensitized preparations and the twitchings following the response of sensitized preparations were abolished by curarine or by atropine, in the same concentrations as were used for the preparations from *R. temporaria*.

DISCUSSION

If the well-known action of quinidine in stopping fibrillation of heart muscle by prolonging the refractory period is recalled, perhaps it might be suggested that the changes which the sensitizers produced, in the response to a single shock applied to the nerve of a preparation which had been treated with any of the eserine-like compounds, except preparation 3393, could be explained in a way other than that advanced at the end of § 1. It might be suggested that the sensitizers shortened the refractory period of the muscle and so favoured repetitive response to a single nerve volley. However, I think that this suggestion can be dismissed, since it has been shown that certain of the sensitizers produce little change in the refractory period of muscle or nerve. Lucas (1910) pointed out that change of calcium ion concentration in the solution bathing frog's sartorius muscle influenced greatly the sensitivity of the pelvic end towards electric stimuli of long duration, whereas the refractory period was influenced little. Bazett (1908) found that the refractory period of frog's sartorius muscle was rather longer in a solution containing little calcium than in a solution containing amounts of calcium chloride comparable with those in Ringer's solution, i.e. the little change that did occur was in the opposite sense to that needed to justify the above suggestion. Bazett's observations are supported by Graham's (1933) work on frog nerve. She found that the refractory period was rather longer after soaking nerve in calcium-poor solution than after soaking in Ringer's solution. Walter and I (1937) found that tetraethylammonium iodide, in small concentration, sensitized nerve to stimuli of long duration, but produced no change in the refractory period.

Feng has examined the responses of toad's (*Bufo-bufo asiaticus*) nerve-sartorius preparations. He found (1937) that eserine caused the muscular response evoked by a single shock to the nerve to become augmented, but that if stimulation was repeated more frequently than once in about 5 min. the augmentation vanished. He found that increase of calcium concentration prevented the augmentation from developing, or reduced it if already developed. Apparently toad preparations differ from frog preparations, firstly, in that eserine causes the response to become augmented without any special sensitization being necessary; secondly, in that the augmented response is more easily depressed by frequent stimulation; thirdly, in that neither twitchings nor after-tension follow the augmented response. Feng (1938) has also described the action of guanidine on toad's nerve-muscle preparations. He used concentrations which were usually higher

(5000–10,000μM) than those which I used to sensitize frog preparations. He found that when the muscle was stimulated by single short shocks applied at its pelvic end, the guanidine did not augment the response. The guanidine did, however, augment the response evoked by a single shock to the nerve, and caused discrete twitchings, which lasted for a minute or more, to follow it. Frequent stimulation caused the after-twitchings to diminish greatly. They were abolished by Ringer's solution of 5–6 times the usual calcium content; they were nearly abolished by curare in concentration almost sufficient to block neuromuscular conduction. Eserine caused the twitchings to be transformed into a smooth after-tension followed by a few late twitchings.

There is a general similarity between Feng's results with guanidine and mine, but since usually we used different concentrations a detailed comparison is difficult. Despite the difficulty there are several points which seem of interest: the very long duration of the after-twitchings in the toad preparations, and the depressant action of frequent stimulation. Also, Feng's experiments upon the combined action of guanidine and eserine may be compared with my experiments with preparations from *R. esculenta*, in which I used higher concentrations of guanidine than usual. I have already drawn attention to the difficulty of deciding how far the after-tension is due to fused asynchronous twitching and how far it is contracture. Feng states that he made one experiment to test the action of atropine sulphate, and found that a concentration of 1 in 7000 did not abolish the after-twitchings produced by guanidine. In more than thirty of my experiments with frog preparations atropine has never once failed to abolish the after-twitchings or after-tension—whichever was present. I am reluctant, therefore, to accept his observation as final, and I would not dismiss the possibility that two processes occur in the region of the nerve ending—one sensitive to atropine and the other insensitive.

SECTION 3

In the previous section it was shown that, when a warm nerve-sartorius preparation which had been soaked in Ringer's solution of one-half the usual calcium chloride content was treated with any of the eserine-like substances, the muscular response evoked by a single shock to the nerve became augmented. Locke (1894) found that within about 20 min. of immersing a nerve-sartorius preparation in an isotonic solution of sodium chloride a single shock to the nerve evoked no response from the muscle, whereas repetitive stimulation of the nerve still caused the muscle to contract. He found that the response of the muscle to direct stimulation was almost unchanged; and that, in the absence of stimulation, the muscle exhibited some spontaneous activity. He also showed that the addition of a small quantity of calcium chloride to the solution restored the effectiveness of nerve stimulation, and stopped the spontaneous activity of the muscle. Cushing (1901) showed that results similar to Locke's could be obtained when the frog's gastrocnemius-sciatic preparation was left *in situ* and perfused with the appropriate solutions. Overton (1902, 1904) found that the addition of potassium chloride, in concentrations comparable with those in Ringer's solution, to the sodium chloride solution accelerated the paralysis to stimulation through the nerve. He found that the paralysis was also accelerated if certain quaternary ammonium salts, known to have curare-like activity, were added instead of potassium chloride. Adrian and Lucas (1912) made experiments with nerve-muscle preparations which had been treated with sodium chloride solution. They found that the effects of shocks applied through one pair of electrodes on the nerve could sum with the effects of shocks applied through a separate pair of electrodes on another part of the nerve to cause contraction of the muscle. This, they pointed out, indicates that local summation in the nerve is of no importance in these experiments. Taking a general view of the observations just cited, it appears that a deficiency of calcium ions affects neuromuscular transmission in some other way, besides the one described in § 2. The experiments of this Section are concerned with that point. Warm nerve-sartorius preparations were treated with calcium-deficient solution, and the course of the changes thereby produced in the form of muscular response to stimulation of the nerve or of the pelvic end of the muscle, at various frequencies, was examined; also, of the further changes produced by subsequent treatment with prostigmine or eserine.

Experimental details

The preparation was mounted in the modified Lucas's trough, and the muscle connected to an isometric spring torsion lever. The arrangements for repetitive stimulation were the same as described in a previous paper (1938), except that a switch was inserted so as to permit, when required, condenser discharge shocks to be used instead of the usual alternate charge and discharge. Single shocks, when required, were condenser discharges.

The calcium-deficient solution was the usual Ringer's solution, but without calcium chloride. Its calcium content was less than 10μM. As a precaution against calcium diffusing out from the inner parts of the preparation and so causing a 'spontaneous' recovery, the calcium-deficient solution was renewed several times during each experiment.

RESULTS

Responses to stimulation of muscle

Test periods of stimulation lasting 10–30 sec. were used. The way in which the form of the response of the Ringer-soaked sartorius muscle varies with the rate of stimulation and the reproducibility of the response have already been described in some detail (Cowan 1938). For the present purpose it is sufficient to state that at stimulation rates between 50 and 180 per sec. the tension rose rapidly to its maximum or almost to its maximum value, remained fairly steady for a few seconds (usually 3–6), and afterwards fell slowly (e.g. figure 8*A*); that so long as rest intervals of 10–15 min. were allowed between the test periods of stimulation, the response, at any one frequency, deteriorated little in 3 hr. The soaking of preparations for ½–2 hr. in calcium-deficient solution caused the response to assume the following form. The tension rose initially to a value 5–20 % smaller than that which had been attained before treatment; for 1–3 sec. the tension remained steady or fell slowly; then, in the next 4–6 sec., it fell to about two-thirds of the initial value; after that the fall continued, but much more slowly (e.g. figure 8*G*). When preparations which had been brought to this state were treated with prostigmine or eserine, in concentrations up to 30μM, for periods up to 2 hr., no greater change occurred in their responses than in the responses of preparations which were not so treated. Whether or not the preparation had been treated with prostigmine or eserine, provided that it had not been in the calcium-deficient solution for more than about an hour, soaking in Ringer's solution of the usual calcium content (with or without prostigmine or eserine) restored the response to such an extent, that it nearly equalled the response of a control preparation which had been kept for the same time in Ringer's solution. If, however, the 'experimental' preparation had been in calcium-deficient solution for more than about 2 hr., the restoration was less complete: after return to the normal Ringer's solution the tension developed in the response was still lower (5–10 %) than in the response of a continuously Ringer-soaked control preparation, and the rate of fall of tension was rather more rapid.

The above-described responses were unaffected when the time-constant of the shocks used was varied between 200 and 500 μsec., and when their strengths were varied between 1 and 1·5 times those needed to evoke maximal response.

Responses to stimulation of nerve

The way in which the form of the response of the Ringer-soaked preparation varies with stimulation frequency has already, like the response of muscle to direct stimulation, been described in some detail (Cowan 1938). For the present purpose the following description is sufficient. In response to stimulation with about 100 shocks per sec. the tension rose to a maximum, usually 1–5 % higher than that evoked by direct stimulation of the muscle at the same frequency; then the tension began to fall slowly, after 2–3 sec. there appeared superimposed on the fall a slow and small rise which lasted for several seconds; passing through a second maximum the tension continued to fall slowly (e.g. figure 8 *C*). The average rate of fall of tension during a 20–30 sec. period of stimulation was rather greater than during direct stimulation of the muscle for the same period. In response to stimulation with about 150 shocks per sec. the tension rose to a maximum which was usually equal to or slightly greater than that evoked by direct stimulation of the muscle at the same frequency; in 2–6 sec. the tension fell rapidly; the fall was succeeded first by a slower (secondary) rise, which continued for several seconds, and afterwards by a very slow fall (e.g. figure 8 *B*).

The soaking of preparations for 5–10 min. in calcium-deficient solution produced the following changes in the responses. Upon stimulating at a frequency of 150 per sec., the tension rose to a maximum which was about 90 % of the maximum attained before the soaking, then fell slowly for 10–15 sec.; during the next 15–20 sec., the tension remained steady, or even rose slightly (e.g. figure 8 *D*). Upon stimulating at lower frequencies, the tension rose to 80–90 % of the maximum attained before soaking, and then fell slowly and smoothly to one-half to two-thirds of the initial maximum. After preparations had been soaked for about another 10 min. in calcium-deficient solution, in response to stimulation with 100 shocks per sec., the tension rose to 50–60 % of the maximum that had been attained before soaking, remained steady for 2–3 sec., and afterwards fell slowly (e.g. figure 8 *E*). The response to stimulation with 150 shocks per sec. was of similar form, but the tension developed was greater throughout.

After preparations had been soaked in calcium-deficient solution for ½–1 hr., as previous workers found, a single shock to the nerve evoked no response from the muscle. When the nerve was stimulated with 150 shocks per sec. the muscle developed no visible tension during the first 1–3 sec., after that the tension rose slowly along a more or less S-shaped curve,

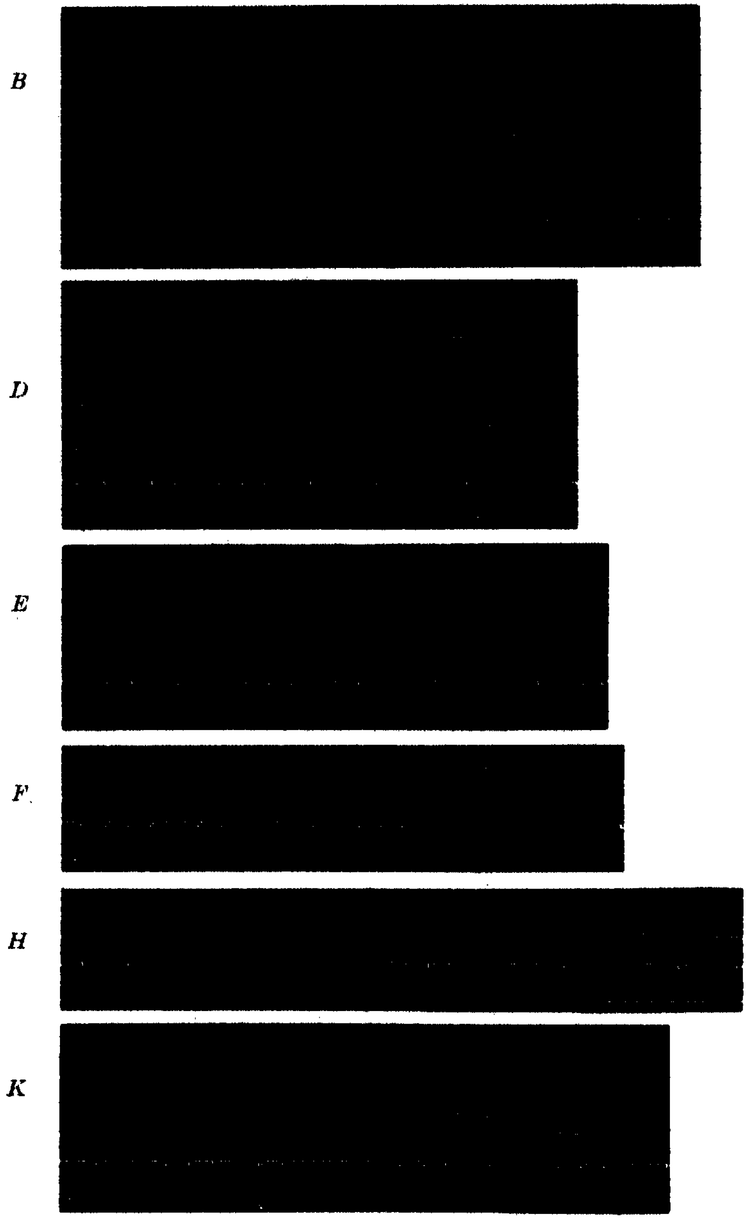
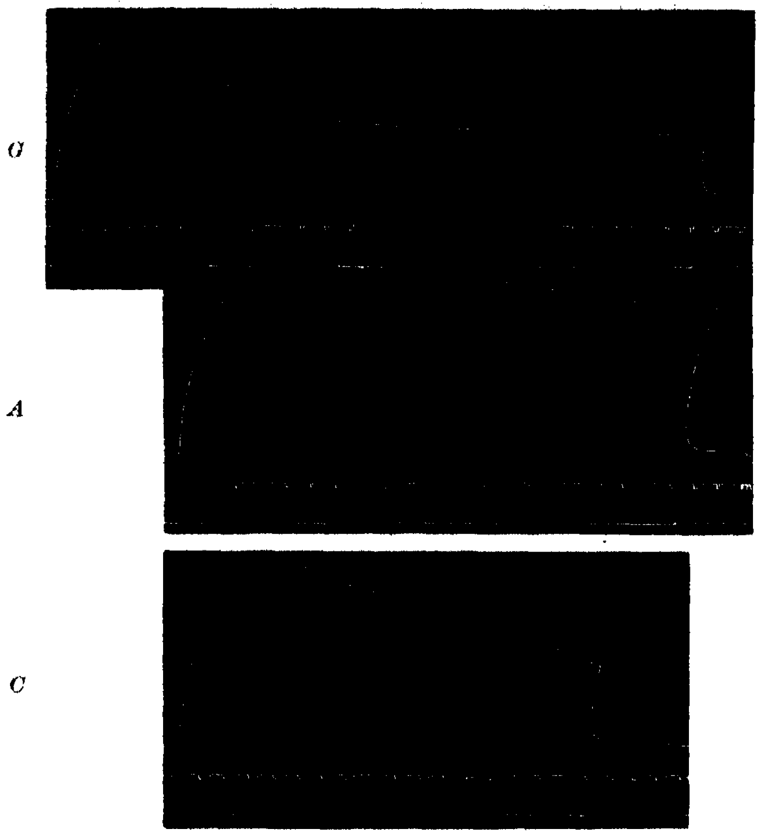


Figure 8. 11. xii. 35. The effect of calcium-deficient Ringer's solution upon the response to repetitive stimulation of the nerve, and of the pelvic end of the muscle, of a sartorius preparation from a warm frog; also, the effect of prostigmine added subsequently.



A, 4.55 p.m., the muscle was stimulated with shocks of time-constant 490 μsec., at a frequency of 150 per sec. whilst the preparation was still immersed in the usual Ringer's solution. *B*, 5.5 p.m., the nerve was stimulated with shocks of time-constant 109 μsec., at a frequency of 150 per sec. whilst the preparation was in the usual Ringer's solution. *C*, 5.35 p.m., the nerve was stimulated in the same conditions as in *B*, but a frequency of 90 per sec.

5.38 p.m., bath drained and then filled with calcium-deficient solution. *D*, 5.45 p.m., the nerve was stimulated with shocks of the same time-constant and at the same frequency as in *B*.

5.48 p.m., bath drained and then re-filled with calcium-deficient solution. *E*, 5.55 p.m., nerve stimulated with shocks of the same time-constant and at the same frequency as in *C*. *F*, 6.8 p.m., nerve stimulated as in *D*.

6.10 p.m., bath drained and then filled with calcium-deficient solution containing 12 μM prostigmine. *G*, 6.35 p.m., muscle stimulated as in *A*. *H*, 6.45 p.m., nerve stimulated as in *D*.

6.53 p.m., calcium chloride solution added to bath to make concentration 0·026 % $CaCl_2$ (2·36 mM.). *K*, 7.10 p.m., nerve stimulated as in *D*.

attaining a maximum in 10–15 sec. Usually the maximum did not exceed 25 % of that attained before the soaking. As stimulation was continued the tension fell very slowly (e.g. figure 8 *F*). If the nerve was stimulated at lower frequencies (70–100 per sec.) the response was of the same form as at the higher frequencies, but the interval which elapsed before the muscle developed any visible tension was longer, the S-shaped rise was slower and the maximum attained was even smaller.

When preparations were brought to the state described in the previous paragraph, and then further treated with calcium-deficient solution containing prostigmine or eserine, the responses to stimulation at various frequencies became slightly improved. Stimulation with about 150 shocks per sec. caused the tension to rise almost immediately to about 10 % of the maximum attained in the responses recorded at the beginning of the experiment; the tension continued to rise slowly, reaching a maximum after 5–10 sec., i.e. earlier than before treatment with prostigmine or eserine. The maximum, however, was not much greater than that attained before treatment. *During further stimulation the tension fell very slowly* (e.g. figure 8 *H*). If the nerve was stimulated at lower frequencies (70–100 per sec.) the form of the response was approximately the same as that just described, but throughout the tension developed was smaller. The time lag between the addition of prostigmine or eserine to the bath and the development of the maximum change was dependent on the concentration of those substances in much the same way as was the time lag in other earlier experiments (Cowan 1938). The improvement of response produced by 100μM solution of prostigmine or a 50μM solution of eserine sulphate was little greater than the improvement produced by a 5μM solution of either substance. I think it probable, although I have made no experiments to test the point, that the slightly greater improvement produced by the more concentrated solutions was due mainly to their shorter time lag, which meant that the preparations had been soaking in calcium-deficient solution for a shorter time.

Soaking the preparation in Ringer's solution of the usual calcium content for 10–15 min. produced an improvement of quite a different order. This happened whether or not prostigmine was also used (e.g. figure 8 *K*). If the preparation had been soaked merely in calcium-deficient solution for less than an hour, soaking for about half an hour in Ringer's solution restored the responses almost to what they had been at the beginning of the experiment. If the preparation had been in calcium-deficient solution for more than about 1½ hr., the restoration was less complete: the tension attained was 60–80 % of what had been attained at the beginning of the experiment; at frequencies between 100 and 150 per sec. the fall of tension from the initial maximum was slower and less pronounced. If the preparation had been treated with prostigmine or eserine, then the form of the restored response was partly dependent on the concentration which had been used. When the Ringer's solution contained prostigmine or eserine, the response first became like that shown in figure 8*K*; after more soaking, the response became like that of a warm preparation which had been treated only with prostigmine-containing Ringer's solution (for details see Cowan 1938).

GENERAL DISCUSSION

It is possible to outline a provisional interpretation of most of the observations described in this paper in terms of the hypothesis that acetylcholine is the transmitter from motor nerve to skeletal muscle. A start will be made with the observations in §§ 1 and 2.

Evidence has already been offered that any of the eserine-like substances caused the muscle of a cold or sensitized preparation to respond repetitively to a single volley in the nerve. This action of the eserine-like substances was probably due mainly to their inhibiting the destruction, by choline-esterase, of the acetylcholine liberated at the nerve endings. Marnay and Nachmansohn (1937*a*, *b*, *c*, 1938) have shown that the choline-esterase in frog muscle is much more concentrated at or near the nerve endings than in other parts of the muscle. The additional possibility that any of the eserine-like substances exerted a sensitizing action upon at least the part of the muscle fibre juxtaposed to the nerve ending, and perhaps upon the remainder of the muscle fibre also, cannot be excluded. Turning now to the after-tension which followed the main response evoked from warm muscle when two shocks were applied in rapid succession to the nerve, it is possible that after each nerve volley a small quantity of ACh. persisted in the region of the nerve endings for a time long in comparison with the absolute refractory period. The action of eserine-like substances in augmenting the after-tension could be attributed to inhibition of the choline-esterase in the region of the nerve endings. Again, the possibility that any of the eserine-like substances also exerted a sensitizing action on the muscle cannot be excluded. To suppose that the after-tension was due to ACh. would accord with its sensitiveness to curarine and to atropine. The action of eserine-like substances in partly transforming into (early) twitchings the after-tension which followed the main response to stimulation of the nerve of a cold or sensitized preparation might be attributed to inhibition of the choline-esterase in the region of the nerve endings. For a third time, the possibility that any of the eserine-like substances exerted a direct action on the muscle cannot be excluded. At present, I can offer no explanation of the action of eserine-like substances in causing twitchings to appear 10–15 sec. after stimulation of the nerve of a cold or sensitized preparation.

Now, interpretation of the observations described in § 3 can be considered. In a previous paper (1938) I suggested: first, that for repetitive stimulation of the motor fibres of a nerve trunk to evoke a tetanic response from a muscle, it would be necessary for the ACh. concentration, in the intervals between volleys, to fall below the threshold needed to initiate excitation from the part of the muscle fibre juxtaposed to the nerve ending, and that the failure of neuromuscular transmission which occurred during rapid repetitive stimulation was due to successive liberations of ACh. following one another more quickly than the agencies responsible for their removal could deal with them; secondly, that the changes observed in the form of the response to repetitive stimulation of the nerve of a warm Ringer-soaked preparation were due to a progressive diminution in the amount of ACh. liberated by each successive nerve volley. I showed that certain eserine-like substances produced changes in the responses to nerve stimulation, which were approximately the same as would have been produced by raising the frequency. I pointed out that those changes were consistent with inhibition of the choline-esterase at the nerve-endings, and/or sensitization of the part of the muscle fibre juxtaposed to the nerve ending. In the experiments just reported, however, the effects of immersing a preparation for 5–10 min. in calcium-deficient solution were essentially opposite to those of the eserine-like substances, i.e. the changes produced in the form of response by a moderate degree of calcium-deficiency, at any of the frequencies examined, were such as might have been produced in the responses of "warm" Ringer-soaked preparations by lowering the frequency. Two possible factors are: first, that the amount of ACh. liberated per nerve volley is reduced in a manner corresponding to the degree of calcium deficiency; secondly, that calcium deficiency diminishes the sensitivity of the part of the muscle juxtaposed to the nerve ending towards ACh. Against the second factor being of major importance are the observations on the relative ineffectiveness of eserine-like compounds in restoring neuromuscular transmission after it had been severely depressed or abolished by calcium deficiency. Recent and more direct observations of Harvey and MacIntosh (1940) also indicate that the second factor is the less important. They found that when the cat's superior cervical ganglion was perfused with Locke's solution from which calcium had been omitted and to which had been added a little eserine, no ACh. could be detected in the perfusate during or after stimulation of the pre-ganglionic fibres.

The effects of immersing a frog preparation in calcium-deficient solution for longer times—15 to 20 minutes—appear to involve at least one additional factor. The ineffectiveness of a single volley, and the reduction of the response to repetitive stimulation to one-half or two-thirds of its normal value, imply that the amount of ACh. liberated by each nerve impulse was reduced below the threshold amount necessary to set up propagated excitation in the muscle fibre supplied. It seems that an ineffective nerve impulse may somehow leave behind a raised excitability in the region of the nerve ending, which may render effective a second and otherwise ineffective nerve impulse. The properties of the after-tension in the responses of Ringer-soaked preparations suggest that ACh. may persist in the region of the nerve endings for some time after a nerve volley. Possibly the raised excitability was due to persisting ACh., but such a conclusion must be regarded as one on which further experimental evidence is needed (cf. Rosenblueth and Morrison 1937; Brown and Euler 1938). The S-shaped form of response which developed after ½–1 hr. in calcium-deficient solution may be consistent with still further reduction of the amount of ACh. liberated by each nerve impulse. In connexion with this last state of the preparation, it must be pointed out that no response appeared until the nerve had been stimulated for 1 sec. or longer, in which time at least 100 shocks were applied; that from the records it is impossible to decide how far the response was propagated excitation in a few muscle fibres and how far it was contracture near the nerve endings; that, consequently it is impossible to decide to what extent the kind of summation referred to above entered into the response.

Feng, and Feng and Shen have also reached the conclusion that the greater the calcium ion concentration in the surrounding fluid the greater is the amount of ACh. liberated by each nerve impulse. Their experiments were made mainly with toad's (*Bufo-bufo asiaticus*) isolated nerve-muscle preparations. The principal observations on which their conclusion rests are: increase of the calcium ion concentration above the normal value in Ringer's solution favoured the development of the failure of neuromuscular transmission which occurs when the nerve is stimulated at high frequency (Feng 1936*a*); increase of calcium-ion concentration restored or partly restored the responses evoked by single maximal shocks from partly curarized preparations; or preparations in which neuromuscular conduction had been impaired by previous stimulation with shocks at the rate of one in 3 sec. (Feng 1936*a*, *b*); increase of calcium ion concentration augmented a neuromuscular contracture which followed the main response to stimulation of the nerve of an eserine-treated preparation for about 1 sec., at a frequency of about 50 per sec. (Feng and Shen 1937).

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The actions of eserine-like compounds upon frog's nerve-muscle preparations, and the blocking of neuromuscular conduction

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The actions of prostigmine, eserine, and the dimethylcarbamic ester of 8-hydroxymethylquinolinium methylsulphate upon the frog's isolated nerve-sartorius preparation have been examined by a method developed by Lucas (1911). With Ringer-soaked preparations from frogs kept at 14–18° C for some days before use the minimum interval at which two shocks applied to the nerve could elicit a summated muscular response was about 20 % longer than the absolute refractory period of the nerve. Any of the above-mentioned compounds prolonged the minimum interval for a summated response, but caused the time at which an extra interpolated shock began to cut down the response to the final shock to become only a little later. Curarine or atropine reversed the prolongation of minimum interval.

By the same method, the actions of the same eserine-like compounds upon preparations which had been treated with Ringer's solution of half the usual calcium content were examined. Either before or after treatment, it was impossible to cut down the muscular response by interpolating an extra shock.

The action of prostigmine upon Ringer-soaked preparations was examined by a method developed by Adrian (1913), involving determination of the rate of recovery of excitability in the motor nerve at the site of stimulation and in the remaining more peripheral part of the preparation. Prostigmine influenced little the recovery process at the site of stimulation, whereas it prolonged the slower and more peripheral recovery process. Curarine reversed the prolongation of the more peripheral recovery.

With Ringer-soaked preparations, during the block of neuromuscular transmission produced by rapid repetitive stimulation of the nerve, the response of the muscle to direct stimulation was reduced. If, however, neuromuscular transmission had been blocked by curarine, stimulation of the nerve did not reduce the response of the muscle to direct stimulation.

In the introduction to the preceding paper I stated my reasons for examining, by the methods of Lucas (1911) and of Adrian (1913), the actions of eserine-like compounds upon frog's nerve-muscle preparations. Certain preliminary work was described in that paper. I shall now outline the methods of Lucas and of Adrian.

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Keith Lucas showed that the minimum interval at which two shocks applied in succession to the nerve of a frog's nerve-muscle preparation could elicit from the muscle two electric responses, or a summated muscular contraction, was longer (generally about 20 %) than the absolute refractory period of the nerve. He found also that if two shocks were applied to the nerve at such an interval that the second produced a summated contraction of the muscle, then a third shock interpolated late enough to set up a second impulse in the nerve, but not late enough to set up a second impulse in the muscle, abolished the effect of its successor (originally the second shock). This, he claimed, was 'Wedensky's inhibition in its simplest form'.

Lucas's observations were extended by Adrian, who made a detailed examination of the effects of varying the strengths of the shocks applied to the nerve. Using two shocks, the first being of maximal strength, he determined the relation between the strength of second shock and the interval which had to elapse between the shocks in order to produce a summated muscular contraction. With some preparations he found that as the strength of the second shock was increased the interval for a summated contraction became shorter, but that once the strength of the second shock exceeded a certain critical value (about 3–5 times threshold) the interval for a summated contraction exhibited no further diminution (e.g. curve *RST* in figure 3A). With other preparations he found no discontinuity in the relation between strength of second shock and interval for summated contraction—the interval diminished progressively as the strength of the second shock was increased up to 10–15 times threshold (e.g. curve *QST* in figure 3A). By varying independently the temperature of the nerve at the site of stimulation and at a region peripheral to it, he was able to show that the second type of curve represented the course of recovery at the site of stimulation; that in the first (or composite) type of curve the approximately hyperbolic part also represented the course of recovery at the site of stimulation, whilst the vertical part indicated that although a strong and early shock could set up an impulse in the nerve, it was nevertheless ineffective if recovery was slower at some more peripheral part of the preparation. Using three shocks and varying the strengths of the second and third, Adrian found that the second shock could render the third ineffective only if the second shock was of such a strength, and was interpolated at such a time, that it set up a second impulse in the nerve whilst the peripheral part of the preparation was still refractory, i.e. only if the second shock was of such a strength and was interpolated at such a time that it fell within the shaded area in figure 3A. He showed that with preparations in which the recovery at the site of stimulation was slower than at the peripheral part this inhibition could not be produced. In establishing the foregoing results with three shocks, Adrian applied the first and second shocks at one pair of electrodes and the third at another pair of electrodes, because he found that, in certain conditions, if all three shocks were applied at the same electrodes a separate and relatively less important inhibition was produced at the site of stimulation; a (strong) second shock following the first too early to set up a propagated impulse in the nerve might inhibit the effect of an otherwise just adequate third shock. He pointed out that this local inhibition was consistent with Gildemeister's (1908) and with Levinsohn's (1910) observations that for a short time after the application of a subliminal stimulus to a previously unstimulated nerve the excitability became depressed locally, reducing the effectiveness of, or making ineffective, a subsequent stimulus. Since then, this change of excitability has been studied by Erlanger and Blair (1931 *a*, *b*) and has been called by them 'the post-cathodal depression'.

Using shocks of short duration to excite nerve, Bramwell and Lucas (1911) found that, within the limits of their method, the absolute refractory period was due to propagated excitation, and not to local effects at the electrodes. More recently, Graham (1933) found that the minimum interval at which two shocks, when applied to nerve through the same electrodes, could evoke two responses was equal to, or slightly greater than, the absolute refractory period due to propagated excitation. There is no reason to suppose that the absolute refractory period of the peripheral part of a nerve fibre is greater than that of the more central part. If, therefore, a nerve-muscle preparation, the whole of which is at a uniform temperature, gives, when examined by Adrian's method, a curve of the composite type, it follows that the slower of the recovery processes occurs at the nerve endings or peripherally to them.

If eserine-like compounds act only upon nerve endings or more peripherally, they would be expected, in terms of Lucas's work, to prolong the minimal interval for a summated muscular response, leaving the absolute refractory period of the nerve unchanged; in terms of Adrian's work, to slow the peripheral recovery process, leaving that at the site of stimulation unchanged. §§ 1 and 2 deal with the experimental testing of these expectations. Some of the results have been summarized in a preliminary communication (1936). § 3 deals with experiments which showed that during the block of neuromuscular transmission produced by high-frequency stimulation of the nerve of a nerve-sartorius preparation the response of the muscle to direct stimulation was also reduced.

MATERIALS

Nerve-sartorius preparations from warm frogs only were used. Except where otherwise stated, the Ringer's solution and other substances were the same as described in the preceding paper. Preparation 3393 was not used: in this paper the expression 'eserine-like compounds' does not include that compound.

SECTION 1

METHOD

Outline. Having regard to the apparatus at my immediate disposal I chose the following method as a simple one for verifying and extending Lucas's observations. With a Ringer-soaked nerve-muscle preparation:

(*a*) the minimum interval at which two shocks applied in succession to the nerve could elicit a summated response (tension) from the muscle was determined;

(*b*) the interval between the shocks was fixed at a value slightly greater than the minimum one, and then the changes produced in the size of the muscular response by interpolating another shock at various times between the other two were examined.

The determinations (*a*) and (*b*) were repeated once or more than once in order to ascertain whether the preparation had reached a steady or very slowly changing state. When such a state had been reached the preparation was treated with Ringer's solution containing the substance whose action was to be examined. The preparation was left undisturbed for a time estimated as sufficient for that action to become well developed, and then the determinations (*a*) and (*b*) were repeated; afterwards they were repeated at intervals so long as appreciable change continued. In some experiments, when a second fairly steady state had been reached, another substance was added to the bath in order that its action subsequent to that of the first could be examined. After another interval, the determinations (*a*) and (*b*) were continued until a third fairly steady state had been reached. Control experiments were made keeping preparations in Ringer's solution and repeating the determinations (*a*) and (*b*) periodically. To provide control experiments of another kind, approximate determinations of the absolute refractory period of frog's sciatic nerve trunks were made before and after treatment with the substance under examination. For these determinations a sensitive galvanometer was used ballistically to detect two electric responses in the nerve.

The above-described experiments were made with warm nerve-muscle preparations. In most instances Ringer's solution was used for the preliminary soaking and subsequent experiment, but in a few instances Ringer's solution of half the usual calcium content was used instead. Preparations which had been treated in the latter way rather than cold preparations were chosen for the experiments involving augmented responses, because their responses did not change slowly as did those of the cold preparations soaked in Ringer's solution containing an eserine-like compound (see preceding paper). For the control experiments with nerve, sciatic trunks from warm frogs were used. The Ringer's solution was buffered to pH 7·2 with phosphate, and contained 0·02 % $CaCl_2$. The reasons for using a phosphate buffer have been discussed previously (Cowan 1938).

Details of experiments with nerve-muscle preparations

The preparation was mounted in the bath described in the preceding paper (figure 1). In some experiments break induction shocks were used to stimulate the nerve, in others condenser discharges of time-constant 50μsec. Both kinds of shock were timed by Lucas's pendulum. The induction shocks were obtained from three coreless coils with their windings at right angles to one another. Their secondary windings were connected to the same electrodes (*P* and *Q* in figure 1 of the preceding paper) in such a way that all the shocks were delivered in the same sense, namely so that the electrode proximal to the muscle (*Q*) was always the cathode. The condenser discharges were obtained by the arrangement shown in figure 2A of the preceding paper. They were also delivered so that the electrode proximal to the muscle was the cathode. The methods of adjusting the strengths of the induction shocks and the condenser shocks were those already described. The muscle was connected to an isometric lever writing on a smoked drum which was moved on by hand after each response had been recorded. In order to make the measurement of the size of the response as easy as possible and to eliminate small variations due either to differences in the friction between the paper and writing point or to changes in the preparation itself, the response to a single shock was recorded alternately with that to each test with two or with three shocks; also, a further precaution, during the determination (*b*) the response to two shocks without the interpolated shock was recorded at intervals.

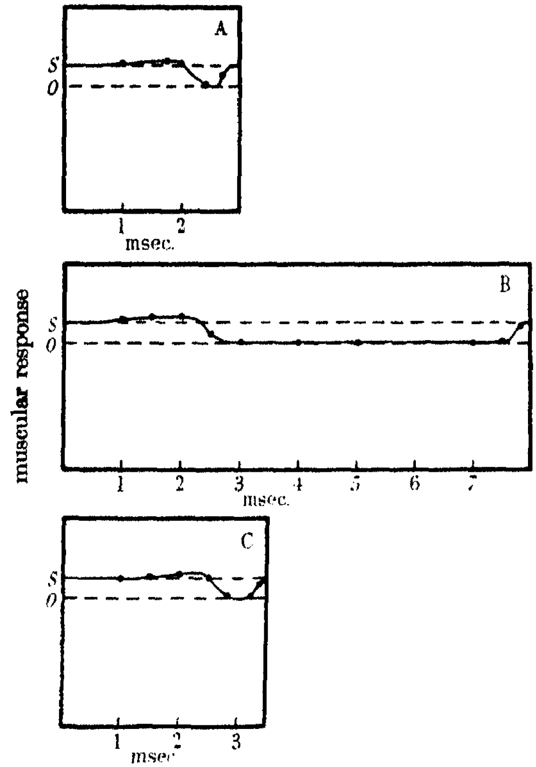

FIGURE 1. 13. v. 36. The action of prostigmine upon the minimum interval at which two shocks applied to the nerve of a frog's nerve-sartorius preparation could evoke a summated muscular response; upon the effectiveness of an additional shock interpolated between the other two. Also, the action of curarine added subsequently. In each diagram the abscissae represent the time interval between stimuli, and the ordinates the height of contraction. The first shock (*a*) was applied at zero time and the second shock (*c*) at the time represented by the extreme end of the abscissa line. The lower broken line (*o*) represents the mean height of a normal contraction evoked by the first shock acting alone; the upper broken line (*s*) represents the mean height of the summated contraction evoked by two shocks (*a* and *c*). The continuous line represents the height of the contraction which resulted when *a* and *c* were sent in as before, and an additional stimulus, *b*, was interpolated between them at the time indicated along the abscissa line.

3.32 p.m., preparation from a warm frog mounted in Ringer's solution.

3.49–4.09 p.m., the minimum interval for a summated response was determined, and the effect of an interpolated shock examined as described in the text. The observations are summarized in diagram A.

4.11 p.m., bath drained and then filled with Ringer's solution containing 3 μM prostigmine.

5.10–5.28 p.m., the determinations of minimum interval, and the examination of the effect of an interpolated shock were repeated. See diagram B.

5.31 p.m., curarine chloride added to bath to make the concentration 0·4 μM.

5.52–6.8 p.m., the determinations of minimum interval, and the examination of the effect of an interpolated shock were again repeated. See diagram C.

Details of the experiments with nerve

The details of the method were those described in a previous paper (Cowan 1938), except that single shocks or shocks timed by a Lucas's pendulum were substituted for repetitive stimulation. The single shocks were break induction shocks or condenser discharges as above.

RESULTS

Ringer-soaked preparations

If the interpolated shock was sent in at the beginning of the interval between the other two, it produced no change in the summated muscular response: if sent in later, it caused the response to be a slightly augmented one: if sent in during the last 20 % of the interval, it reduced the response. The results of this part of an experiment are summarized in figure 1 A.

In the experiments with preparations which were soaked in Ringer's solution for about 3 hr. the minimum interval for a summated response increased—generally by 10–30 %. The time at which the interpolated shock began to reduce the response also became later, by 10–20 %.

The action of eserine-like compounds upon Ringer-soaked preparations

Treatment for 1–2 hr. with a solution containing 0·06 μM prostigmine prolonged the minimum interval for a summated muscular response by 50–100 %. The time at which the interpolated shock began to reduce the response became 10–20 % later. The interpolated shock was effective in reducing the response if sent in any time between then and the end of the summation interval. Treatment for 1–1½ hr. with a solution containing 3 μM prostigmine prolonged the minimum interval for a summated response by about 200 %, i.e. from about 2–2·5 to 5–8 msec. As in the experiments with weaker prostigmine solutions, the time at which the interpolated shock began to reduce the summated response became 10–20 % later (figure 1 B). Treatment with solutions containing prostigmine in greater concentrations than those stated above prolonged the minimum interval to an even greater degree. The time at which the interpolated shock began to reduce the response became little if at all later than it did after treatment with the weaker prostigmine solutions.

Results qualitatively indistinguishable from those described above were obtained when eserine or Substance 38 was used instead of prostigmine.

The action of curarine or atropine upon preparations which had been treated with an eserine-like compound

If a preparation whose minimal interval for a summated response had been prolonged by prostigmine or eserine were subsequently treated for ½–1 hr. with curarine chloride in concentration 0·2–0·4 μM, the minimum interval became shortened again to approximately what it had been at the beginning of the experiment, when the preparation was soaking in pure Ringer's solution. The time at which the interpolated shock began to reduce the response remained unchanged or became slightly later. Changes of this kind are shown in figure 1C.

The action of atropine sulphate in concentration 1 in 10,000 upon prostigmine-treated preparations was indistinguishabl from that of curarine chloride in concentration 0·2–0·4 μM.

Experiments with nerve

In different experiments with nerve the galvanometer deflection due to the action current response to a single shock ranged from about 1·5–3 mm. The position of the spot on the scale was steady and could easily be read to within 0·2 mm. Whilst admittedly the sensitivity was insufficient to detect a small second response in the nerve, and consequently any small changes of refractory period, it was sufficient to ascertain that prostigmine, curarine or atropine, in the concentrations stated above, did not prolong the absolute refractory period by more than 30–50 % of its initial value.

Experiments with preparations soaked in Ringer's solution of one-half the usual calcium content

The minimum interval for a summated response was 2–2·2 msec.—perhaps a little shorter than in the experiments with preparations soaked in Ringer's solution of the usual calcium content. An interpolated shock sent in early between the other two produced no change in the summated response; sent in later, it produced a very slight augmentation. When the preparation was treated with modified Ringer's solution containing 3 μM of prostigmine, the response evoked by a single shock to the nerve became augmented and was followed by twitchings, as described in the preceding paper. The minimum interval was prolonged by less than 20 %. An additional shock interpolated at any time during the summation interval produced no appreciable change in the response. Subsequent treatment with curarine chloride or atropine sulphate in concentration just sufficient to abolish the augmentation of response to a single shock, and the after-twitchings, produced no change in the minimum interval; nor did either substance cause the interpolated shock to produce any change in the response.

DISCUSSION

The observations with Ringer-soaked warm preparations are in agreement with the earlier observations of Lucas. Lucas showed that the time at which the interpolated shock began to reduce the muscular response was the earliest time at which a second shock following a first could evoke a second electric response from the nerve. He also showed that, corresponding to the slight augmentation of muscular response which occurred when the interpolated shock was sent in during the earlier part of the summation interval, there occurred a slight augmentation of the electric response of the nerve. He suggested that the augmentation occurred because the second shock, falling on the nerve fibres whilst they were still refractory, set up local excitations which enabled the third shock to excite a few fibres which it would otherwise have been unable to excite. Recently Katz (1937) has offered evidence for a local response in frog nerve; Hodgkin (1938) found that in non-medullated, crustacean nerve a local electric response could be detected just before the end of the absolute refractory period. It may be that Lucas's suggestion is correct, but the possibility of a polarization effect at one of the membranes of the nerve trunk cannot yet be excluded.

My finding, that the time at which the interpolated shock began to reduce the muscular response became little or no later after treatment with any of the eserine-like substances, curarine, or atropine, than it became after treatment with Ringer's solution alone, implies that these substances have very little action upon the absolute refractory period of nerve. This is supported by the rough determinations of refractory period of sciatic nerve trunks. It appears, therefore, that the actions of the above-mentioned substances, on the muscular response to nerve volleys, are effected at the nerve endings or peripherally to them. Before we can regard this conclusion as firmly based, however, there are two possibilities to be disposed of. They are possibilities to which Adrian gave attention in his earlier work. One, already mentioned at the beginning of this paper, is that the interpolated shock exerted a local inhibitory action on the nerve. The other is that the first and second shocks set up volleys in the motor nerve fibres, whereas the interpolated shock sets up a volley in some other group of fibres of the mixed nerve, which recover more quickly than the motor fibres. Although the above-described results indicate that neither possibility is at all probable, I thought it well to try to obtain more direct evidence by Adrian's method.

SECTION 2

METHOD

For these experiments it was necessary to apply shocks to the nerve of a nerve-muscle preparation at two different points. To this end the partitions in the bath shown in figure 1 of the preceding paper were removed and replaced by others arranged as shown in figure 2. By leading in current through the non-polarizable zinc-zinc sulphate half-cells *X* and *Y* shocks could be applied to the central part of the nerve; by leading in current through the half-cells *W* and *V* shocks could be applied at a point about 20 mm. nearer the muscle. One, two, or three break shocks were obtained by means of the Lucas's pendulum and three coreless coils, as described earlier. The shocks were so delivered that the cathode was the electrode nearer the muscle, i.e. *W* or *Y* was the cathode.

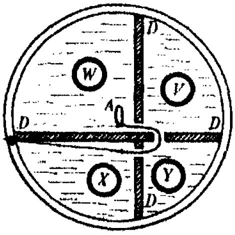

FIGURE 2. Plan of vertical type of bath modified so as to permit stimuli to be applied, through two independent sets of electrodes, to the nerve of a nerve-sartorius preparation, at two places with a distance of about 20 mm. between them. *A*, nerve-sartorius preparation in position. *D*, *D*, *D*, *D*, vertical partitions. *W*, *V*, *X* and *Y*, zinc-zinc sulphate half-cells for leading current into the compartments. Other details were the same as described in figure 1 of the preceding paper.

Only Ringer-soaked, warm preparations were used. In order to simplify description of an experiment, I shall anticipate the results to the extent of stating that the curves obtained were all of Adrian's first (or composite) type. The main steps were as follows. First, the threshold of the motor nerve fibres for a shock applied through the more central electrodes was determined. As Adrian has pointed out, the threshold of the motor fibres is a convenient unit in which to express the strengths of the shocks used in any one experiment, and to compare the results of different experiments. The second step was to fix the strength of the shock at a slightly supra-maximal value, and then, by sending in a second shock through the same electrodes, at various times after the first shock, to determine at each of those times the minimum strength of second shock needed to evoke a summated muscular response. The third step was to repeat the determination of threshold of the motor nerve fibres. The fourth step was to readjust the first shock to a slightly supra-maximal strength and then applying the second shock, adjusted to a strength of about fifteen times threshold (i.e. 3–5 times maximal), at the more peripheral electrodes, to set the interval between the shocks at a value slightly greater than the minimum one for a summated muscular response. The fifth step was to send in, through the central electrodes at various times between the other two shocks, an interpolated shock, and to determine at each of those times the minimum strength needed to inhibit the summated response of the muscle. The sixth step was to repeat the determination of threshold of the motor nerve fibres. After these determinations had been completed the bath was drained and then filled with Ringer's solution containing prostigmine. and the preparation was left undisturbed for 1½ hr. At the end of that time the steps one to six were repeated. This done, a small amount of curarine chloride was added. After another half hour the steps one to six were again repeated.

Control experiments were made with preparations which were kept in Ringer's solution for the whole time.

RESULTS

Three experiments and two control experiments were made.

Whilst the preparation was in Ringer's solution the relation between the minimum interval for a summated response and the strength of second shock was that summarized by the full lines in figure 3A. The minimum interval diminished with increase of shock strength up to about 5 times threshold, but was unaffected by further increase. The dotted line shows the relation between the time at which the interpolated shock was sent in and the least strength needed to reduce the summated muscular response. Figure 3B shows the same two relationships as figure 3A, but after the preparation had been treated with 3 μM prostigmine solution. The minimum interval for a summated response was little changed with strengths of second shock up to about twice threshold; further increases of strength did not shorten it. The limiting value was about 7 msec. The strength-time relation for inhibition by the interpolated shock was changed as follows: weaker and later shocks became effective; shocks of moderate strength remained effective at about the same times as before treatment; strong shocks became unable to inhibit quite so early as they had done before treatment. After the preparation had been treated with curarine chloride in concentration 0·4 μM the two relationships became those summarized in figure 3C. The limiting value of the minimum interval (4·1 msec.) was reached with a strength of second shock less than 3 times threshold. Strong or moderately strong interpolated shocks inhibited the muscular response at the same time as before the curarine, late and weak shocks became unable to inhibit.

In the control experiments the limiting value of the minimum interval for muscular summation increased by 10–20 % in about 3 hr.; also, the time at which a strong interpolated shock could inhibit muscular summation became about 10 % later (see figure 4A, B).

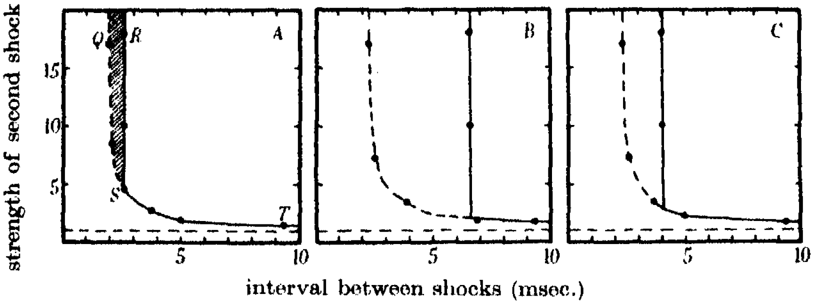

FIGURE 3. 19. v. 36. The action of prostigmine upon summation and inhibition curves determined by Adrian's method. For further explanation see text. Also, the action of curarine added subsequently.

3.22 p.m., preparation from a warm frog mounted in Ringer's solution.

3.33–4.0 p.m., summation and inhibition curves determined. See diagram A.

4.3 p.m., bath drained and then filled with Ringer's solution containing 3 μM prostigmine.

5.40–6.6 p.m., summation and inhibition curves determined a second time. See diagram B.

6.8 p.m., curarine chloride added to bath to make concentration 0·4 μM.

6.37–7.1 p.m., summation and inhibition curves determined a third time. See diagram C.

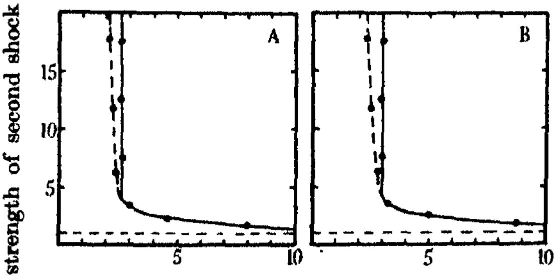

FIGURE 4. 21. v. 36. A control experiment in which a preparation from a warm frog was soaked only in Ringer's solution. Summation and inhibition curves determined by Adrian's method. Further explanation in text.

2.48 p.m., preparation mounted in Ringer's solution.

3.30–3.54 p.m., summation and inhibition curves determined. See diagram A.

3.57 p.m., bath drained and then re-filled with fresh Ringer's solution.

5.49–6.11 p.m., summation and inhibition curves determined again. See diagram B.

DISCUSSION

Since the hyperbolic part of the curve relating the interval between two shocks to the strength of second shock necessary to evoke a summated response was changed little more, if at all, by prostigmine than by Ringer's solution, it is to be concluded that prostigmine influenced very little the (local) recovery process in the motor nerve fibres. The shifting of the vertical part of the curve towards longer intervals indicates that prostigmine slowed the already slower recovery process occurring at the nerve endings or peripherally to them. The finding that the strength-time curve for inhibition of the muscular response by an interpolated shock was continuous with the hyperbolic part of the summation curve, both before and after preparations were treated with prostigmine, is, I think, good ground for presuming that the former curve was the remainder of the curve representing the recovery of the motor nerve fibres. If so, the nerve volley set up by the interpolated shock travelled in the motor fibres. The observation that prostigmine changed the inhibition curve very little, if at all, supports the conclusion that prostigmine influenced very little the recovery process in the motor nerve fibres.

Now that the objections raised in § 1 have been disposed of, it is convenient to gather together certain conclusions. In warm, Ringer-soaked preparations, on the one hand, apparently the setting up of an impulse in a muscle fibre, by a nerve impulse, is followed by a block at the nerve ending or at some part peripheral to it. The block lasts longer than the absolute refractory period of the nerve fibre. During the block a second impulse arriving at the nerve ending is not only ineffective, but prolongs the block. Although prostigmine does not influence appreciably the recovery process in the nerve, nor the response of a muscle to a nerve impulse (apart from causing an after-tension to appear, as described in the preceding paper), it prolongs greatly the block which occurs at the nerve ending or peripherally to it. Curarine or atropine, in small concentration, can shorten the block which has been prolonged by prostigmine. In sensitized preparations, on the other hand, the setting up of an impulse in a muscle fibre, by a nerve impulse, is not followed by a block. Nor does treatment with prostigmine, in small concentration, cause such a block to appear. The muscular response becomes augmented, as described in the preceding paper.

SECTION 3

The experiments on warm, Ringer-soaked preparations, described in this and the preceding paper, suggested that the block which followed the passage of a volley from the nerve to the muscle would extend into the part of the muscle near the nerve endings; that with preparations which had been treated with an eserine-like substance the block would also extend into the muscle, whereas with preparations which had been treated with curarine the block would not extend into the muscle. The general structure of the sartorius preparation makes it very suitable for an experimental test of these suggestions. If the block extended into the muscle, it was to be expected that impulses starting from the pelvic end would fail to travel the whole length of the muscle fibres and that consequently the size of the response would be reduced.

Experiments have been made in two ways. In both of them repetitive stimulation at frequencies higher than 25 per sec. was used because the records so obtained can be easily understood. One way was to record the effect upon the tension developed by the muscle when the nerve was stimulated for a few seconds at a frequency high enough to produce neuromuscular block whilst the pelvic end of the muscle was being stimulated continuously. The other way was to record the tension developed by the muscle in response to stimulation of the pelvic end for a few seconds whilst the nerve was being stimulated at a frequency high enough to produce neuromuscular block.

Before going on I must make it clear that Feng (1936*b*, 1937*a*) has already published the results of some experiments nearly all of which were made with toad's nerve-sartorius preparations (*Bufo bufo asiaticus*). As most of my observations with frog's preparations differ little from Feng's corresponding observations I have endeavoured to make my description as short as possible, although not so short that it cannot be understood without reference to Feng's papers. Also, I have included a few observations which have not been recorded by Feng.

When I made my experiments I was ignorant of Feng's work, although the first paper cited had been published nearly three months earlier. I remained ignorant of Feng's work until about a month after publication of the second paper cited. Since then Feng has published a paper (1937*b*), which deals with the relation between local heat production in muscle and the blocking of propagated excitation during block of neuromuscular transmission.

EXPERIMENTAL DETAILS

The modified Lucas trough and isometric spring torsion lever mentioned in § 1 of the preceding paper were used. The following precautions were taken against interference between the shocks applied to the nerve and those applied to the muscle. The preparation was so arranged that the longest axis of the nerve was perpendicular to the plane containing the two longer axes of the muscle. Four zinc-zinc sulphate half-cells were used to lead in the stimulating currents—two for the nerve and two for the muscle. The half-cells carrying the current for the muscle were placed along the longest axis of the muscle, and the half-cells carrying the current for the nerve along the longest axis of the nerve. Repetitive stimulation of the nerve was effected by alternate charge and discharge of a condenser by rotating commutator (Hill 1934). Stimulation of the muscle was effected by a similar but quite separate apparatus. The effective resistances shunting the nerve and the muscle condensers were the same as in earlier experiments on repetitive stimulation (1938).

RESULTS

If, whilst the muscle of a Ringer-soaked preparation was giving a steady or slowly declining response to stimulation of the pelvic end at a frequency between 70 and 200 per sec., the nerve was also stimulated at a frequency low enough for neuromuscular transmission to be well maintained (40–80 per sec.), no change occurred in the response. If, however, the muscle was being stimulated at low frequency (30–50 per sec.) and then the nerve was stimulated at a frequency lying at the higher end of the range mentioned above, the response suffered a transient augmentation which never exceeded 2 % (figure 5A–C).

In the converse type of experiment to that just described, stimulation of the muscle whilst the nerve was being stimulated at a frequency between 40 and 80 per sec. produced no change in the response.

In contrast to the foregoing results, if, whilst the muscle was giving a steady or slowly declining response to stimulation at a frequency between 70 and 200 per sec., the nerve was stimulated at a frequency high enough to produce almost immediate failure of neuromuscular transmission (usually 180–200 per sec.; see Cowan 1938), the response sank rapidly to 60–90 % of its normal size: it recovered again only a little less rapidly if, after a few seconds, stimulation of the nerve was discontinued. If the muscle was stimulated at lower frequencies (30–50 per sec.), the diminution of response produced by stimulation of the nerve was often preceded by a small and transient increase. In experiments of the converse type, stimulation of the muscle whilst neuromuscular transmission was blocked evoked a diminished response.

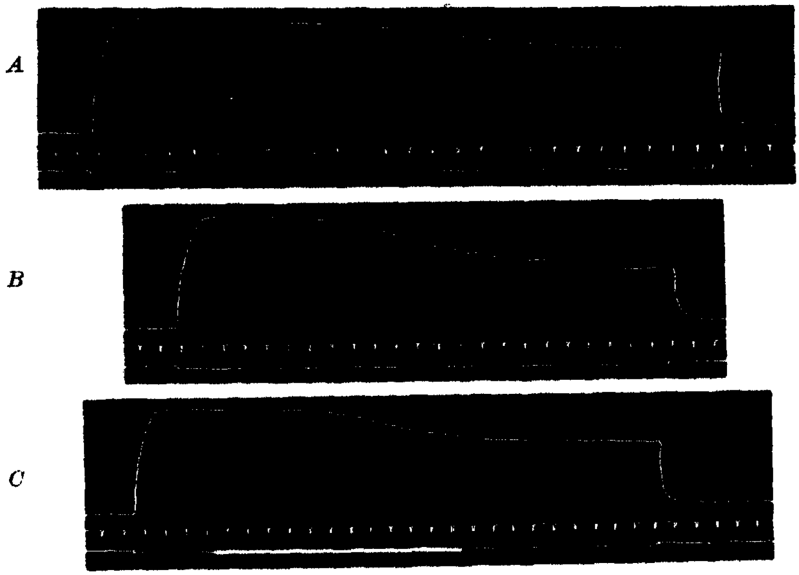

FIGURE 5. 24. vi. 36. The slight and transient augmentation of muscular response produced by stimulating the nerve of a sartorius nerve-muscle preparation from a warm frog at a frequency of 75 per sec. whilst the pelvic end of the muscle was being stimulated at a frequency of 34 per sec. The time markings are seconds. Depression of the signal line indicates approximately the beginning of stimulation, and the subsequent rise indicates the end of stimulation.

A, 3.19 p.m., muscle of Ringer-soaked preparation stimulated as above with shocks each of time-constant 490 μsec.

3.32 p.m., bath drained and then refilled with Ringer's solution.

B, 4.7 p.m., nerve stimulated as above with shocks each of time-constant 109 μsec.

4.10 p.m., bath drained and then refilled with Ringer's solution.

C, 4.56 p.m., the muscle was stimulated continuously for about 25 sec. The nerve was also stimulated for the period indicated by the thickening of the signal line.

Treatment of Ringer-soaked preparations with prostigmine or eserine lowered the frequency at which the above-described diminution of muscular response to direct stimulation occurred. The responses of preparations which had been treated with prostigmine or eserine differed from those of Ringer-soaked preparations in two additional respects. These differences were more marked the higher the frequency at which the nerve was stimulated, and the greater the concentration of prostigmine or eserine with which the preparation had been treated. One difference was that, upon discontinuing stimulation of the nerve whilst the muscle was being stimulated, the response recovered more slowly than did the response of Ringer-soaked preparations. The other difference was that if, whilst the muscle of a prostigmine-treated or eserine-treated preparation was being stimulated, the nerve was stimulated for 3–7 sec., allowed to rest for 5–7 sec., stimulated for another 3–7 sec., and then allowed to rest again, the response recovered more slowly after the second period of nerve stimulation than after the first. The recovery of the muscular response became still slower after a third period of nerve stimulation. This is shown in figure 6C: after the first period of nerve stimulation the response recovered in about 1·1 sec., after the second period in about 1·7 sec., after the third period in about 3·8 sec.

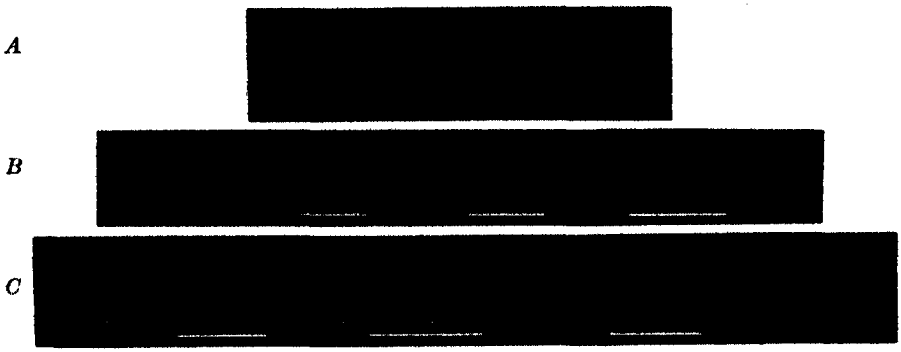

FIGURE 6. 16. vi. 36. The reduction of the response to direct stimulation of muscle produced by stimulating the nerve of a prostigmine-treated, warm nerve-sartorius preparation at a frequency high enough to cause rapid failure of neuromuscular transmission. The time marks, the depression and rise of the signal line, have the same meanings as in figure 5.

5.26 p.m., bath drained and then filled with Ringer's solution containing 6 μM prostigmine.

A, 6.13 p.m., pelvic end of muscle stimulated with shocks each of time-constant 490 μsec. at a frequency of 25 per sec.

B, 6.26 p.m., the nerve was stimulated continuously for about 40 sec. with shocks each of time-constant 109 μsec. at a frequency of 75 per sec. At times indicated by thickening of the signal line the muscle was also stimulated at a frequency of 25 per sec.

6.30 p.m., bath drained and then refilled with more Ringer's solution containing 6 μM of prostigmine.

C, 6.45 p.m., the muscle was stimulated continuously for about 50 sec. at a frequency of 25 per sec. At times indicated by thickening of the signal line the nerve was also stimulated at a frequency of 130 per sec.

With preparations which had been treated with curarine in a concentration such that neuromuscular transmission was blocked or almost blocked, stimulation of the nerve whilst the muscle was being stimulated produced no change in the response.

The results described in this section were uninfluenced by varying the time-constant of the shocks applied to the muscle from 200 to 500 μsec., by varying the time-constant of the shocks applied to the nerve from 50 to 250 μsec., by varying either stimulus voltage between 1 and 1·5 times that needed to evoke maximal response.

GENERAL DISCUSSION

The hypothesis that acetylcholine is the chemical transmitter from motor nerve to voluntary muscle is, I think, consistent with the foregoing observations. In an earlier paper (1938) I suggested that the blocking of neuromuscular transmission resulting from a rapid series of nerve volleys might be attributed to accumulation of acetylcholine at or near the nerve endings. From the observations described in §§ 1 and 2 it might be concluded that if a single volley is set up in the motor nerve fibres of a warm preparation, then at the end of the absolute refractory period of those motor fibres a quantity of acetylcholine sufficient to block a second volley remains at or near the nerve endings. Part of the localized acetylcholine may diffuse into adjacent regions in the muscle, part may be destroyed by the choline esterase at or near the nerve endings (Marnay and Nachmansohn 1937*a*, *b*, *c*, 1938). The observations described in § 3 indicate that the block extends into the muscle fibre (cf. Osterhout (1932) on the blocking of propagated excitation in *Valonia* cells; Dale and Gasser (1926) on the blocking of propagated excitation in frog muscle by ACh.), and that therefore diffusion is by no means a negligible factor. The additional observations that curarine shortened the block following a single nerve volley, and that rapid stimulation of the nerve while transmission was blocked by curarine in no way interfered with contraction of the muscle in response to direct stimulation, are evidence that acetylcholine and not potassium ions were primarily responsible for the block in untreated preparations. It is possible, however, that in the untreated preparations the acetylcholine itself caused a secondary liberation of potassium ions, which reinforced and prolonged the block.

The differences between Feng's observations on the effect of rapid stimulation of the nerve in blocking propagated excitation in the muscles of toad's preparations and my corresponding observations on frog's preparations are quantitative rather than qualitative. At present I will attempt no more than to state some factors which may account for the differences. Feng and Ting (1938) have shown that in toad's sartorius muscle, as in frog's, the choline esterase is more concentrated at or near the nerve endings than in the other parts. Marnay (1938) has offered evidence that toad's muscle contains only half as much choline esterase as does frog's muscle. Feng (1936*a*) has shown that toad's sartorius muscle ordinarily responds with a tetanus at the make of a constant current, but that after a preliminary soaking of the muscle in Ringer's solution its response becomes more like that of warm frog's muscle. Lucas found that the minimum current gradient needed to set up propagated excitation in toad's, Ringer-soaked sartorius muscle (1907) was smaller than that needed for frog's, Ringer-soaked muscle (1908). It is possible also that the amount of acetylcholine liberated at the nerve endings is smaller in toad's than in frog's preparations.

My sincere thanks are due to Mr E. B. Verney and to Professor J. H. Gaddum for facilities which enabled me to carry out this work in their respective laboratories; to Professor E. D. Adrian for the loan of a Lucas's pendulum with calibration curve; to Professor F. R. Winton for the loan of electrical apparatus.

I am indebted to Roche Products, Ltd., and Hoffmann-La Roche and Co., Ltd., Basle, for supplies of eserine-like compounds. I am also indebted to the Government Grants Committee of the Royal Society and to the Research Grants Committee of the Senate of the University of London (Thomas Smythe Hughes and Beaverbrook Funds) for allowing me to retain apparatus which had been purchased with grants from them and used for other earlier work.

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Address of the President Sir William Bragg, O.M., at the Anniversary Meeting, 30 November 1940

Many events conspire to make the past year notable in the history of our Society. Reference has been made to the majority of them in the Annual Report of Council, usefully supplemented by the Notes and Records which we continue to owe to our past Treasurer, Sir Henry Lyons. I do not propose to speak of them in detail, but on this occasion it does seem fitting to give further attention to one or two general matters of lasting interest.

One of these is personal. Fellows will have noted the long list of those whom we have lost, and the great names which the list contains. I have felt as I have been reading it that I have turned over the last leaves of a chapter that stands by itself. The present generation is quick to honour the names of J. J. Thomson and Oliver Lodge, but they cannot remember, as we older men can, the brilliant years when these men and their contemporaries were writing the chapter's first pages. What they wrote was eagerly read, their lectures were heard with rapt attention; they were the pioneers, and the scientists of that time, nearly half a century ago, streamed after them. All that is now a memory. The years have slipped away since their work was done, and we now look back on it and see it as a separate entity, a noble event in the history of science, and of British science in particular.

There is no vestige of sadness in such a retrospect, nor any trace of feeling that our pride must be founded only on what has passed. I am sure that all those who like myself can recall the long years, and compare those that have gone by with those that are still ours, will say happily and proudly that our young men of to-day are maintaining in full force the tradition that they have received. They are writing a new chapter; and it is a chapter of a novel importance because as they extend our record of the facts of Nature they find themselves compelled at the same time to consider a new problem, the relation of those facts to society and to the government of nations. Let me express my admiration of the willingness, vigour and ability with which the newer generation gets to work.

This same novelty is enlarging the range of work of our Society and is a second matter to which we are compelled to give serious attention. Our Fellows have constantly given their services to public interests; it has often been pointed out that they are to be found in association with almost every department of government. But this year new moves have been made which may, and I hope will, lead to developments of the highest importance. The Report contains a notice of the recent formation of a Scientific Advisory Committee over which Lord Hankey presides, with a reference which in effect directs it to consider the advances of science in their relation to national welfare. The Committee reports to the Cabinet through its chairman. A Committee of similar nature but lesser scope was set up a few months ago to consider the scientific aspects of the food policy of the Government: it consists of well-known authorities on nutrition, agriculture and economy, with myself as chairman. This Committee reports to Lord Privy Seal, and so to the Cabinet Food Policy Committee over which Lord Privy Seal presides. The *significant feature* of these committees is their close and direct association with the Cabinet, the central body that governs our nation. Hitherto scientists have been appointed man by man to various Government Departments so that they might act as useful items in departmental machinery. The new committees are not parts of any executive body and have no executive power of their own. They exist to make recommendations, which must of course be practical and take full account of difficulties of execution. But they are not hampered by traditions, nor by set habits; they have time and freedom to consider the whole field of scientific knowledge and its possible influence on practice. The Scientific Advisory Committee, the more important of the two, is particularly well fitted to watch all occasions and opportunities for the employment of science in the service of the nation, and also for the continuous encouragement of that employment. The President of the Society and the two principal Secretaries are in close touch with every branch of science; through the Fellows of the Society which they serve they have a unique view of scientific progress. The three Secretaries of the principal Research Councils of the Government, dealing with industry, agriculture and health, are in close touch with the chief national activities.

Thus a great opportunity is opened after long expectation; and the Royal Society is largely responsible for the development of that opportunity. We hope that no hindrances from without may interfere with the Society's task, and we are determined that there shall be no lack of energy from within.

We remember that it is science itself, not scientists, that we are trying to lift to the high places. In that respect our movement is not selfish. We do not claim that scientists shall be entrusted with authority because they are scientists: we do claim that authority shall be exercised in the light of a knowledge which grows continuously, and with continual effect on politics, on industry, and on thought itself. If at present the only way to bring this knowledge into use is to treat scientists as consultants, let us take that way. But we shall be taking the better way if in all ranks of the State, and especially in those that have authority and set an example, we can arouse a general appreciation of the position, and a constant understanding watchfulness on the increase of knowledge and the uses that are made of it and can be made. It cannot be said that the general aspect of the nation towards the increase of knowledge is satisfactory. Science has become an integral part of our educational system, yet the changes that have been made are often ridiculously like the casting of sacrifices to following wolves. Science is not a devouring monster, but a means of service; it is a knowledge, gained by an irresistible tendency of man to examine his surroundings. It may be rightly or wrongly used. There is a prime danger if those who are in the position to use it rightly shut their eyes to its presence and its power, like an army which relies on bows and arrows when its enemies know how to use machine guns.

It is not universally nor even sufficiently understood how important natural knowledge has become. It is true that in a vague way the nation is brought by the happenings of war to guess at the meaning of scientific research in every kind of enterprise. But still it would be difficult for most people to grasp the significance, much less the meaning of the description of a fact like this: that the R.A.F. could not carry out its operations without the knowledge resulting from the studies of cathode-rays and electrons made by our physicists, which is equivalent to saying that by this time we might well have lost the war. Similar cases of cause and consequence could be quoted in numbers; they happen to be found more readily in relation to the sciences that deal with inorganic materials than those that deal with organic processes, and the military demand for physicists has been great because they are wanted to put physical discoveries into practice. But this discrimination is only accidental and temporary, and in fact the whole range of science is equally concerned.

Since experimental science has assumed such a commanding influence on all our affairs, so that we run the risk of great perils if we take no account of it, and leave its uses to others, let us say less well disposed than ourselves, and, on the other hand, have opportunities of great benefit if we use it rightly, it becomes a first duty to direct our steps accordingly. Just as in former times schools and colleges were founded to train men for the service of Church and State, in ways which were appropriate to that high end, so now we have to see to it that the men are produced by our educational systems who can appreciate and act up to a new state of affairs. This can be done without jettisoning any of the fine instruction which has been a proud feature of our older systems.

I think that this is not essentially a matter of the rearrangement of school time tables, or the building of scientific laboratories, though such tactical methods must have their due consideration. This is a personal matter, as has been the case with every great human movement. We have not to force the use of new tools, but to encourage and develop a new appreciation and a new attitude. Our best method, as ever before, lies in our own actions. If we, in the continually increasing contacts of scientists with public affairs, can show that we have something of great value to contribute, and that we give it freely, placing our individual interests below those of a greater purpose; if we try to understand the motives and principles of those whom we meet who may not see our vision just as we may fail to appreciate theirs, then by so doing we have the best chance of bringing about the changes that we desire. It is the personal contact of the scientist, especially with those who are charged with duties to the nation, that is the moving force. That is where these new associations of science with government may mean so much, and shall mean it, if our devotion can achieve its purpose.

This afternoon I leave the Presidential chair. I have deeply appreciated the honour that has been paid to me by my election to it, and I want to thank with all my heart the officers of the Society, the members of Council, and the permanent officials who have helped me to fulfil its duties.

CHARLES BARROIS (1848–1939) was elected a Foreign Member of our Society in 1913. He belonged to an industrial family in the north of France, and came to England as a young man to study English methods of manufacture. He became interested in geology, and particularly the geology of Great Britain and Ireland. His work in this field won him a doctor's degree at the Sorbonne. He completed investigations of high importance into the difficult geologies of Normandy, Brittany, northern France and Lorraine. He was head of the geological department of the University of Lille. He was well known in this country, especially to the geologists by whom he was held in great esteem. The Geological Society of London awarded him the Wollaston Medal in 1901.

WILFRED TROTTER (1872–1939) was one of the most distinguished surgeons in this country. He was especially a master of the surgery of the brain and of the thyroid gland. He studied at University College and won the Gold Medal and University Scholarship in Surgery of the University of London in 1899. His outstanding skill and knowledge gave him a wide practice as a consultant. After being Consulting Surgeon to the King from 1928 to 1932 he became Sergeant Surgeon to the King in 1932 and held that office under three Sovereigns.

In 1934 Trotter gave up private practice in order to devote himself to work at University College Hospital and to the training of young men in the Medical School. He was a fine teacher. He added to his wide knowledge and his technical skill a precision of thought and a love of thoroughness which had a profound influence on his students.

Trotter took a great interest in qualities of the human mind as well as in the structure and functions of the human body. In 1915 he wrote in the *Sociological Review* on the herd instinct, and in later years he wrote on the mentality of nations at war. He studied the functions of nerves and the causes of pain.

Trotter was for many years a member of the Medical Research Council. He was elected to our Society in 1931, and twice served on our Council: on the latter occasion he acted as a Vice-President.

EDMUND NEVILLE NEVILL (1847–1940) was an able student of dynamical astronomy. He was especially interested in the perturbations of the moon due to the influence of the planets. In 1882 an observatory was built in Durban and he was appointed its first Director, arriving in Durban just in time to make observations of the transit of Venus on 6 December. He carried on the appropriate observational work, and at the same time pursued the theoretical researches which had established his reputation. Nevill was one of the original Fellows and a member of the first Council of the Institute of Chemistry. From 1888, until his retirement in 1912, he combined the post of Government Chemist and Official Assayer for Natal with that of Government Astronomer. He was elected a Fellow of the Royal Astronomical Society in 1873, and a Fellow of the Royal Society in 1908.

GILBERT MORGAN (1870–1940) was a great chemist. His skill, knowledge, versatility and energy were to no small degree responsible for the present sound position of British chemistry. In particular, he did much to promote the useful co-operation of laboratory and factory. Morgan was a student of Meldola when he was working on dyestuffs, and through his influence obtained a position as research chemist in the dyeing industry.

It is interesting that during a long research on the dihydroxynaphthalenes he prepared a representative of the substances afterwards known collectively as bakelite, but its future great usefulness was not then anticipated since it had no application in the production of artificial colours. In 1919 he was appointed to the Mason chair of Chemistry in Birmingham. His greatest work was begun when in 1925 he became the first Director of the Government Research Laboratory at Teddington. He made an unqualified success of the new enterprise. He designed its structure and organized its work: and the fact that so much useful knowledge has proceeded from it, especially in the last war months, is proof of his foresight and of the soundness of his management.

Morgan was a hard worker; he was kind and helpful to other workers, and a valuable counsellor in all chemical business. He was awarded the medal of the Society of Chemical Industry, of which he was President from 1932 to 1933. In 1915 he became a Fellow of the Royal Society. He was knighted in 1936.

JOHN HENRY MICHELL (1863–1940) made very important contributions to the subjects of elasticity and hydrodynamics. After an early education in Melbourne, Michell went to Cambridge. He was bracketed senior wrangler in the Mathematical Tripos in 1887, and was elected to a Fellowship at Trinity College. His work included the solution of a number of problems related to the behaviour of structures under stress. He discussed hydrodynamical problems also, and among other investigations he was responsible for that relating to the 'highest wave' in water.

He was for some years a lecturer in the University of Melbourne, and subsequently professor of mathematics. He retired in 1928. He was a hard worker, who kept up his devotion to his special subjects until the end of his life. He was an excellent teacher and writer. A former student of his, Professor Massey, writes that his modesty and good nature, combined with his remarkable intellectual qualities and willingness to assist in the smoothing out of difficulties, however trivial, endeared him to his students.

He became a Fellow of our Society in 1902.

SYDNEY JOHN HICKSON (1859–1940) was a distinguished zoologist devoted to the study of sedentary forms of life. In his early years at school he acquired a liking for the study of natural science, which after his first class in the Cambridge Tripos of 1881 led to his establishment in the laboratory of Michael Foster and F. M. Balfour. There he completed a classical research on 'The eye and optic tract of insects'. In 1882 he became demonstrator to Moseley at Oxford and in 1888 was a deputy professor there. He spent a year in zoological and anthropological studies in Celebes. For a few years he lectured at Cambridge and in 1894 was appointed to the Beyer professorship of zoology at Manchester. There he accomplished the great work of his life. While he pressed on with his zoological work, he also played a great part in the work of the University as a whole; he took a great part in the promotion of social life of the University, especially on the women's side. He was enthusiastic in the promotion of nature teaching in schools and in the development of school museums. He was a great figure in the educational world of Lancashire.

In 1926 he retired to Cambridge and, though he could now look back on a long life devoted to scientific work, he continued his zoological researches with even increased mastery, until he died in the early months of this year. In 1895 he was elected a Fellow of our Society.

ROOKES EVELYN BELL CROMPTON (1845–1940) was one of the most notable and honoured figures of electrical engineering, because on the one hand he was associated with its first lumbering efforts and on the other because he himself was the author of many of the advances which gave to it its present adaptability and enormous power. It was an extraordinary experience to meet in recent years this wonderful old man and to remember the variety of his experiences; they went so far back as service in the Crimean war, for which he held the medal and Sebastopol clasp. He was for some years after that at Harrow, which he left in 1860. He joined the Rifle Brigade when he was eighteen and served in India. He was seconded for special service as superintendent of the Government Steam Train Department. He backed his pioneering work on mechanical transport by his construction of 'The Blue Bell' which had an average speed of four miles an hour, but was followed by other 'Blue Bells' that moved somewhat faster. From 1878 he devoted himself to the new problems of electrical engineering, and was associated with other pioneers, John Hopkinson, Willans, Swan and others, in the first enterprises of a new industry. He lived to see the world-wide application of designs and conceptions with which he himself had been associated at the beginning of his long life. He was naturally elected on many occasions to the presidency of great engineering institutions. He was elected to our Society in 1933.

WALDEMAR CHRISTOFER BRØGGER (1851–1940) was a very distinguished geologist and mineralogist. His main work was concentrated on the rock formation and contents of the Oslo country and its fiords. So thoroughly did he describe this very rich district that it became a well-known field of study to which Brøgger himself often acted as guide. This work won for him the Murchison Medal of the Geological Society of London. From 1900 onwards he occupied the chair of mineralogy and geology at the University of Oslo. His enthusiasm for scientific research, his great ability in his own field, and his considerable administrative powers, gave him a leading place among Norwegian scientists. He has been described as one of the most prominent men of science whom Norway has ever produced. He was elected to a Foreign Membership of our Society in 1902.

The Earl of CRAWFORD AND BALCARRES (1871–1940) became a Fellow of our Society in 1924. So strong and able had been his support of all that the Society stands for that the Council gladly exercised on his behalf their right to elect a very limited number of persons on the ground that their election would be of signal benefit to the Society. Lord Crawford was educated at Eton and Magdalen College, Oxford. For some years he sat in the House of Commons; he served in the war of 1914–18 as private and as lieutenant. In 1916 he was recalled to England to take office as President of the Board of Agriculture and Fisheries, and subsequently held other offices in the Government. He was a member of the Cabinet in 1916 and 1922. He was Chancellor of the University of Manchester, a Trustee of the British Museum, of the National Gallery, of the National Portrait Gallery, and he was chairman of the Fine Art Commission. In all the duties which fell to him in these and other offices he took infinite pains to make his services effective. He drew largely on his extensive knowledge of archaeology, of the history of art, and of the humane studies in general. The nation has reason to be grateful to him for the good taste which has been displayed in many public activities, and we of the Society in particular for the warm support which he gave to the advancement of knowledge of every kind.

THOMAS HEATH (1861–1940) was, as Sir D'Arcy Thompson has described him in an understanding memoir, one of the most learned and industrious scholars of our time. He was one of the last to win double honours at Cambridge, where he took a first class in the Classical Tripos and was high in the list of Wranglers. He entered the Treasury in 1884 and rose to be one of the two Permanent Secretaries.

All his leisure was spent on the subject of Greek mathematics, to which his studies at Cambridge supplied an apt introduction. He became a master in this field of knowledge. He wrote books, which are accepted as standards, on the work of Diophantus, Apolonius of Perga, Archimedes, Euclid and other Greek mathematicians. They are remarkable for their completeness and accuracy.

In addition to his public service at the Treasury and subsequently as Comptroller-General of the National Debt Office, he was one of the Cambridge Commissioners under the Universities of Oxford and Cambridge Act of 1923, and a member of the Royal Commission on Museums and Galleries from 1927 to 1929. He was elected to our Society in 1912, and served twice as a member of Council. He was knighted in 1909.

PATRICK LAIDLAW (1881–1940) is best known probably for his work on canine distemper, and on influenza. He was educated at the Leys School and St John's College, Cambridge, completed his medical course at Guy's Hospital and graduated in medicine in 1907. From 1909 to 1914 he was a member of the staff of the Wellcome Physiological Research Laboratories, and during that period carried out in conjunction with Dale a standard piece of research on histamine. He then joined the staff of Guy's Hospital as Lecturer on Pathology, but in 1922 returned to a research post at the National Institute for Medical Research.

Laidlaw's work with Dunkin on distemper led to the successful immunization of dogs, for which surely he will long be remembered with gratitude. It should further be remembered that this was a relatively early study of a virus disease from all aspects, including the means of producing immunity to the infection. The methods which Laidlaw evolved in the course of it played, accordingly, an important part in the astonishingly rapid developments which have since taken place in this field. Prominent, again, among these was the further discovery made by Laidlaw, with Andrewes and Smith, of a virus as the primary infective agent in epidemic influenza, which has opened entirely new lines of attack on that common enemy of mankind. He was counted to be one of the best of modern pathologists, whose contributions to knowledge spread also into physiology and biochemistry, and were only represented in part by what was published in his own name. His death at so early an age is a very serious loss.

Our Society elected him to its Fellowship in 1927, and recommended him for a Royal Medal in 1933. He was knighted in 1935.

HERBERT ALBERT LAURENS FISHER (1865–1940) was a distinguished scholar and statesman whom our Society was glad to elect as a Fellow under the Statute which allows the Society to invite to its membership persons who have rendered conspicuous service to the cause of science or are such that their election would be of signal benefit to the Society. When President of the Board of Education from 1916 to 1922 he instituted a number of reforms of the highest importance. Not all of them are in full application as yet, but he planned a road which our National Educational System has already entered and must continue to follow. Fisher's reputation as a writer will doubtless rest mainly on his famous *History of Europe*, but many other works of his show his great erudition and painstaking accuracy. Besides his term of public duty as a Cabinet Minister, Fisher spent some years as Vice-Chancellor of the University of Sheffield, and he was Warden of New College, Oxford from 1925 until his death. He was a Governor of the B.B.C., was a Trustee of the British Museum and filled many other important posts. He was given the O.M. in 1937; and joined our Society in 1920.

ALFRED CORT HADDON (1855–1940) was in his school-days intended for his father's firm of printers and type founders, but his interest in zoology was so compelling that he was in the end sent to Cambridge where he worked at zoology under F. M. Balfour. For a short time he served as a demonstrator in comparative anatomy, and then was appointed to the professorship of zoology in the Royal College of Science in Dublin. He became secretary of the Dredging Committee which worked off the south-west coast of Ireland. He was sent to Torres Straits in 1888. The year which he spent there roused those interests in anthropology which governed the work of the rest of his life. He was one of our first anthropologists, and not only that but also one of the leading pioneers of a new science. In 1900 he became a lecturer in physical anthropology. The importance then attached to the subject was measured by his salary of £50 a year. In 1898 the Cambridge Expedition to Torres Straits was formed with Haddon as leader. Though its preliminary purpose was the rescue of knowledge concerning the decaying culture of a primitive people, its main intention and its principal work was the study of the people's psychology, as a part of that social anthropology now become so important.

Haddon's own share in the work of the expedition led to his election to our Fellowship in 1899, and to a Readership in Ethnology at Cambridge. His subsequent life was devoted to the development and appreciation of his subject in many ways, by his collection illustrating the arts, tools and customs of primitive people, by his many writings and by his encouragement of anthropological enquiry in all fields, physical, social and technical.

Augustus Edward Hough Love (1863–1940) was famous for his researches and his writings on the elasticity of materials, and particularly for his studies in geodynamics. As a boy he was taught at the Wolverhampton Grammar School, from which he went as a sizar and subsequently a scholar to St John's College, Cambridge. He was second wrangler in 1885, and Smith's Prizeman in 1887. He was elected to a Fellowship at St John's in 1886, and became Sedleian Professor of Natural Philosophy in Oxford in 1899.

Love was an indefatigable worker who gave all his energies to the prosecution of the subjects in which he was so deeply interested, and of which he became an acknowledged master. His investigation of the forms of waves which may travel along the surface of the earth as it is constituted has been of great importance in the theory of earthquake movements. His *Mathematical Theory of Elasticity* is a standard work of the first class both in matter and in style. The same care and perfection appeared in his lectures. He contributed greatly to our knowledge of electromagnetic waves.

Love became a Fellow in 1894 and twice served on our Council. He was awarded a Royal Medal in 1909 and the Sylvester Medal in 1937. He received the de Morgan Medal in 1926 from the London Mathematical Society.

Jocelyn Field Thorpe (1872–1940) was educated at King's College, London, at the Royal College of Science and at Heidelberg, where he studied organic chemistry under Victor Meyer. From 1895 to 1910 he was at Owens College, Manchester, with W. H. Perkin as research fellow and lecturer. For the next four years he worked at Sheffield as a Research Fellow of our Society. In 1914 he became professor of organic chemistry at the Imperial College.

Thorpe was a devoted student of organic chemistry. His researches were the foundation for some of the most important ideas and methods of to-day. More than that, he was, in his generosity and sympathy, a fine leader for the young men who came to work with him, and have helped on so many instances to place British chemistry in its present strong position. He laboured to draw chemical efforts together, and to make them effective in the national service. He served as chairman of many committees and societies: the Indian Chemical Services Committee, Safety in Mines Research Board, Explosives in Mines Committee, the Chemical Society, and the Institute of Chemistry, and he was an active member of the Chemical Defence Committee, the Dye-stuffs Development Committee, and other bodies. He edited the Supplement to Sir Edward Thorpe's *Dictionary of Applied Chemistry*, and has recently been engaged in the production of a new edition of it.

Thorpe became a Fellow of our Society in 1908; and was awarded the Davy Medal in 1922. He was knighted in 1939.

ARTHUR HARDEN (1865–1940) spent the greater part of his working life on the chemistry of the yeast cell and so made a material contribution to the new science of biochemistry. He studied at Owens College, Manchester, and at the University of Erlangen. He was a lecturer at Owens from 1888 until in 1897 he came to London to take charge of a section of the British Institute of Preventive Medicine, afterwards to become the Lister Institute. It was as head of the biochemical section of the Institute that he directed for thirty years a concerted attack upon bacterial enzymes, bacterial metabolism, and kindred subjects. He played also a valued part in extending our knowledge of the vitamins.

In 1929, for his work on alcoholic fermentation, Harden shared with von Euler the Nobel Prize in chemistry. As editor of the *Biochemical Journal* for twenty-five years he had a great influence on the development of biochemistry in Britain. In 1930 his contribution to the *System of Bacteriology* was a very able summary of a section of this subject.

Harden was knighted in 1936. He was elected to our Society in 1909 and was awarded the Davy Medal in 1935.

ALFRED FOWLER (1868–1940) won his way from local schools at Keighley in Yorkshire to the Royal College of Science in South Kensington, which he entered in 1882 with the aid of one of the exhibitions founded in the name of the Duke of Devonshire. He then became assistant to Sir Norman Lockyer, an association which was a main feature of his life. Their accurate and extensive work added largely to our knowledge of spectra. It must have been a source of great satisfaction to Fowler to watch the growing use of his spectral measurements as modern physics showed their full meaning, and the importance of their indications. In due time a demonstratorship in astrophysics was succeeded by an assistant professorship, and then by a professorship which he held from 1915 until in 1923 he became one of the first Yarrow professors of the Royal Society. To the end of his life he continued his researches on the spectroscopic side of astrophysics and won international reputation. He was elected to the Royal Society in 1910, was awarded a Royal Medal, and was the recipient of many foreign honours.

Fowler was a man of great charm. His colleagues, his friends and his students loved him for his continuous willingness to help and the warmth of his encouragement, and they admired him for the perfection of his work.

ALFRED GIBBS BOURNE (1859–1940) was a biologist of distinction whose chief services were given to Madras and the educational institutions of the Madras State. He was educated at University College and the Royal School of Mines, London. He was an assistant to Ray Lankester from 1879 to 1885; the last two years of this period was spent in research at the Zoological Station at Naples. In 1885 he was appointed to the chair of biology in Presidency College, Madras; he was Registrar of the University, Botanist to the Madras Government, Director of Public Instruction and held in succession a number of other important public posts. He was Director of the Institute of Science at Bangalore from 1915 to 1921. He wrote on a wide range of biological subjects and rendered valuable aid to Indian science. He was made K.C.I.E. in 1913. He was elected a Fellow in 1895.

OLIVER JOSEPH LODGE (1851–1940) will be remembered for the highly important contributions which he made to the theory and the use of electric waves. His work in this field was done in the closing decades of last century, though for many subsequent years he was an active and influential leader in physical science.

Lodge was able to enter the University of London mainly as the result of unaided study, but having made his entry he was quick to develop his exceptional abilities. He had the advantage of doing experimental research under Frankland, Guthrie and Huxley. After he had filled various minor posts he became professor of physics in the University of Liverpool in 1881. In 1900 he became Principal of the University of Birmingham.

Lodge was first attracted to the study of electromagnetic waves by Maxwell's development of Faraday's researches. When Herz in 1885 first devised experiments which demonstrated the existence of these waves, Lodge at once took up the new line of research, examined the conditions for their emission and their receipt, and in particular showed the value of correct tuning. Modern radio transmission owes very much to his theoretical investigations and to his experimental skill. This is well known, and yet his name would be even more closely associated with the development of wireless if he had not given the greater attention to research. It is a very interesting fact that he persisted in giving the ether a reality which is now considered to be unnecessary; and if his views are no longer accepted, it is still true that his conceptions assisted himself in his work, and were of no little use to those who followed his lead. He was a magnificent lecturer; his matter and style were of the first order, and his expositions derived further force from his impressive personality.

He was a Fellow of our Society for fifty-three years. He was knighted in 1902.

CHARLES FREWEN JENKIN (1865–1940) was a distinguished engineer who made important contributions to our knowledge of the properties of materials, especially of such as are used in aeroplane construction. He was educated at Edinburgh and Cambridge. The first twenty years of his life were spent in mechanical engineering practice; he was at one time Assistant to Works Manager at the L.N.W.R. works at Crewe, and held other posts of like nature. In 1908 he became Professor of Engineering Science at Oxford, and held this post until 1929.

In 1920 the Aeronautical Research Committee published a 'Report on Materials of Construction' which was the result of many years' work by Jenkin and his assistants. The new emphasis on lightness of construction required a careful analysis of all that was known respecting the strength and form of materials and the purposes for which they were intended. Jenkin demonstrated the inadequacy of previous knowledge and directed enquiry into the channels that had become necessary. A vivid account of the position is given in his Presidential Address to a section of the British Association in 1920. He emphasized the need for studying the processes of 'fatigue', and the effects of surface cracks and other defects superficial and internal. He extended our knowledge of corrosion fatigue. He made a number of other interesting enquiries with matters of physical and engineering science, such as earth pressure, refrigeration, loud speakers, an 'Oxford Astrolabe' and so on. A man of great energy, and a keen student, he has given good service to engineering science. He was elected a Fellow in 1931.

The DUKE OF BEDFORD (1858–1940) was one of the main supporters in this country of zoological studies, by his writings, by his services to zoological enterprises and by his generous expenditure on behalf of zoological work. He formed a magnificent collection of animals at Woburn Park and studied very thoroughly the conditions of acclimatization in Britain of animals foreign to the country. He was President of the Zoological Society from 1899 to 1936 and was responsible for many of the great advances made by the Society during that period. He took a special interest in the Aquarium and in Whipsnade.

He served in the Grenadier Guards in Egypt in 1882 and was A.D.C. to Lord Dufferin when Viceroy of India. He served also in the regular army from 1914 to 1919. He was A.D.C. to King Edward VII and King George V. He was President, for a period, of University College Hospital, also of the Imperial Cancer Research Fund, and he was for some years a Trustee of the British Museum. Our Society elected him to its Fellowship in 1908, being anxious to show its deep appreciation of the benefits which zoological science had received at his hands.

JOSEPH JOHN THOMSON (1856–1940) was one of the greatest men of science of our time. He had the affection and esteem of the whole world of culture. When he died, a few weeks ago, testimonies of his personality and appreciation of his work appeared in every journal, and every scientist remembered that in some way or other he was in his debt.

The very number and fullness of the descriptions of the man and his work make it superfluous for me to relate the details of his life. We all are in fact well acquainted with them. As Fellows of the Royal Society of which he was a most distinguished member, we think gratefully of his services to those principles and purposes which are our essential care. He was entirely the type of man of whom our Society can most be proud. He was a great physicist, whose insight and perseverance revealed new modes of research, leading to some of the most wonderful advances of our time. He was an inspiring teacher. He gave fine service to his University, to his College, Trinity, of which since 1918 he had been Master, to our Society and, in many ways not always well known, to the national Government. It was all recognized. Numerous honours came to him from learned bodies in all parts of the world; he was awarded the Nobel Prize in Physics in 1906, he was knighted in 1908, and the King awarded him the Order of Merit in 1913.

He was our President from 1915 to 1920, and at many other times a member of our Council.

HAROLD CARPENTER (1875–1940) had a world-wide reputation as a metallurgist, whose acknowledged position as a leader was due to the very great contributions he had made both to theory and to practice. At Oxford he was a scholar of Merton and obtained a first class in the Natural Science Honours Schools. He studied also at Leipzig, and was given the Ph.D. He was a research fellow and demonstrator at Owens College, Manchester, from 1898 to 1901, and professor of metallurgy at Manchester from 1906 to 1913. When the National Physical Laboratory was founded he was appointed to be head of the Chemical and Metallurgical Departments: the most important and fruitful years of his life were spent as professor of metallurgy in the Royal School of Mines.

Carpenter's chief work was concerned with the study of the formation and properties of alloys. He laid a foundation for this in a series of original and accurate researches. Perhaps the best known of his achievements is the method of producing single crystals of metals, by means of which it has been possible to throw a new light on the relation between structure and properties. These labours have fortunately coincided with, and to no small extent have been responsible for, the growth in the knowledge of alloy structure which has had a most important influence not only on metallurgical science but also on its application to British industry.

Carpenter was in turn President of all the great metallurgical institutions of Britain. He was frequently asked for advice by the Government and by industrial concerns. He was an excellent teacher. He has left behind him an abiding influence on metallurgical science.

Carpenter received many acknowledgements, British and foreign, of the esteem in which he was held. He became a Fellow of our Society in 1918, and was knighted in 1929.

CHARLES GABRIEL SELIGMAN (1873–1940) was educated at St Paul's School and St Thomas's Hospital, where he became house physician and director of the Clinical Research Laboratory. His first contact with anthropology was made when in 1898 he joined the Cambridge Expedition to the Torres Straits under Dr Haddon, of whom I have already spoken to-day. Although he went back to his medical research work when the expedition returned, and was appointed to direct the clinical laboratory at his old hospital, he had become so interested in ethnological study that he devoted to it the rest of his life. In 1904 he revisited New Guinea as a leader of the Daniels Ethnographic Expedition. In 1908 he went to Ceylon, accompanied by his wife, who also was an enthusiastic worker on the same lines, in order to gather knowledge about the Veddas. From 1909 onwards the two ethnologists were engaged with the concurrence of Government in the study of the peoples of the Sudan. All these researches were of the greatest importance to a new and growing subject. In 1910 Seligman was appointed lecturer in ethnology in the University of London, and in 1913 professor of that subject at the London School of Economics. He took a leading place in anthropological and ethnological societies and meetings in this country. His full and able descriptions of his observations and discoveries are standard works. He became a Fellow of our Society in 1919.

Robert Abbott Hadfield (1858–1940) was a great engineer and metallurgist. His skill, energy and directive power made the Hecla works one of the most important and distinctive of the steel works of modern times; his success was largely due to the devoted attention which he gave to scientific research into the processes of his industry. In 1875 he entered the laboratories of the works founded by his father: his attention was quickly drawn to the several attempts which had been and were then being made to add to the properties of manufactured steel by the addition of other substances, particularly manganese, and in 1882 he produced his famous 12% manganese steel, which was non-magnetic, became softer and tougher on quenching from high temperatures and was extremely resistant to abrasion. Later he was instrumental in developing the silicon and aluminium steels which have been of great use in the construction of electrical transformers and machinery.

Hadfield was thus successful in the development of great enterprises which have been of immense value to this country. He attached high importance to research, and was generous and persistent in its support. His liberality was exercised in many other ways. He was elected a Fellow of our Society in 1909 and was a frequent contributor, alone or in collaboration with others, to the Royal Society *Proceedings*. He was knighted in 1908, and received a baronetcy in 1917.

Henry Head (1861–1940) was a great neurologist who made important contributions to the knowledge of sensation, especially in relation to the skin and the spinal cord. He studied also the problems of aphasia. His work was original and led the way to much of the research of the present day.

Head was educated at Charterhouse and at Cambridge, where he was a scholar of Trinity College. He studied also at the German University of Prague and at the University of Halle. He worked for his medical degree at University College, London. He became a Fellow of our Society in 1899 and was knighted in 1927.

Taking his place worthily in succession to Ferrier, Jackson, Horsley and Gowers, great British neurologists and Fellows of this Society, Head gave most of his working years to the study of diseases, and particularly those of the nervous system, by the experimental method which he had learned as a physiologist. His enthusiasm led him to study on his own person the process of recovery from an experimentally inflicted nerve lesion, and he had a special gift for communicating his enthusiasm to others. During the last 20 years of his life a disease deprived him progressively of the power of movement, but left him to the end full of interest in research and in its further promotion. He was knighted in 1927.

VITO VOLTERRA (1860–1940), who at the time of his death had been a Foreign Member of the Royal Society for thirty years, was one of the foremost mathematicians of the age. After graduating at Pisa and holding chairs in that University and in Turin, he was appointed in 1900 Professor of Mathematical Physics in the University of Rome, and later a Senator of the Kingdom, the Italian Government making use of his ability in the conduct of affairs in many missions to other countries. In the Senate during the years 1914–15, he was one of the strongest supporters of Italy's entry into the war on the side of the Allies.

In 1931, having refused the oath of allegiance then imposed on University Professors by the Fascist Government, he was deposed from his chair. His last years were spent chiefly in Paris.

He will be remembered in the history of mathematics as the creator of the theory of integral-equations 'of Volterra's type', the theory of functionals, and the theory of integro-differential equations, and as the greatest of the pioneers in the application of mathematics to biological problems, especially the fluctuations in the numbers of animal species living together. His contributions to mathematical physics, particularly to the theory of electricity, were also of the first importance; and it was most often from physical problems that he derived the first suggestions for his discoveries in pure mathematics.

ARTHUR NEVILLE CHAMBERLAIN (1869–1940) was elected to our Society in 1938. The tributes to his life and work have been so many and so appreciative, being based on the universal admiration of himself and of his labours, and are still so fresh in our memories that it would be useless for me to add to them. I will say only that we have been proud to have his name on our roll, and that we remember with gratitude his support of learning, and his strivings that the world should be governed by reason and good will.

FREDERICK WALLACE EDWARDS (1888–1940) was born at Fletton, Peterborough. From the Cambridge County School, where he early displayed a marked attraction to botany and zoology, Edwards entered Christ's College, Cambridge, and graduated in 1909; he took the degree of Sc.D. in 1931.

In 1910 he joined the staff of the Department of Zoology in the British Museum, as an assistant in the Entomological section. In 1937 he was promoted to be Deputy Keeper of the Department of Entomology which had been created in the interval. He was given charge of the mosquitos, the crane flies and the British collection of Diptera. Edwards flung himself wholeheartedly into his task and from the very first his published work showed a mastery of his subject and a great critical faculty.

In addition to his purely museum work, Edwards made two successful expeditions, the first to Patagonia, Chile and the Argentine which produced a far-reaching account of the Diptera of these regions. The second expedition was made from a desire to investigate the relationships existing between the faunas of the isolated high mountain groups of East Africa and was concentrated mainly on Mount Ruwenzori. The first results of this last expedition have also been published.

Edwards has described some 2000 new species, but his descriptions were only incidental to constructive taxonomic work on a large scale and his output of published work is represented by over 270 titles.

Ernest William MacBride (1866–1940) was a very able zoologist who adhered strongly to the 'Lamarckian view of the inheritance of acquired characters'. He was educated at Queen's College, Belfast and St John's College, Cambridge, where he was a Foundation Scholar. He studied also at the Biological Station at Naples. At Cambridge he came under the influence of Adam Sedgwick, and was led to an extensive study of the embryology of the Echinodermata: in after years he contributed an important article on this subject to the *Cambridge Natural History*. He held the Strathcona chair of Zoology at McGill University from 1897 to 1909; from 1913 to 1934 he was Professor of Zoology at the Imperial College, London. He did useful public service in connexion with Fishery Research and other zoological enquiries; and wrote a number of important books and papers.

He became one of the leading authorities on embryology, to which his early work on the Echinodermata had introduced him. The strong views on Lamarckism were held with great vigour and much experimental work and argument were spent in the defence of his position against opposition. He became a Fellow of the Society in 1905.

Awards of Medals, 1940

The COPLEY MEDAL has been awarded to Professor PAUL LANGEVIN.

Professor Langevin was one of the band of young pioneers who, in the closing years of the last century, were engaged in exploring the field which had been opened up by J. J. Thomson's discovery of the electron. He spent a year (1897–1898) at the Cavendish Laboratory, and his Paris Dr ès Sc. thesis (1902) is dedicated to J. J. Thomson. The thesis, a notable one, was entitled *Recherches sur les Gaz Ionisés.* It dealt mainly with the recombination and mobilities of ions, their coefficients and the relations between them. It is a standard work on this subject. In it he also devised and applied new and elegant methods of measuring these quantities which were an advance on all their predecessors and have not since been improved upon to any appreciable extent. Related to this, and coming later, were important contributions to the theory of the diffusion of gaseous ions and its relation to ionic mobilities.

Langevin's greatest achievement is the foundation of the electron theory of magnetism. The theories of paramagnetism and of diamagnetism are still very much as he made them and left them over thirty years ago.

There are few branches of contemporary physics which he has not illuminated and improved by his writings, and his work generally has the qualities of breadth, clearness, elegance and completeness which stamp the master.

He has had a great international influence. He has been a prominent figure at all the meetings of the conferences arranged by the Institut International de Physique Solvay since they started in 1911. On the death of Lorentz he was chosen to succeed him as President of the Institut.

He was awarded the Hughes Medal in 1915 and elected a Foreign Member of the Society in 1928.

The RUMFORD MEDAL is awarded to Professor KARL MANNE GEORG SIEGBAHN.

Professor Siegbahn, member of an old Swedish family, is, in the field of X-rays, what Rowland was in the field of ordinary optics sixty years ago. He has introduced high precision into X-ray measurements. For example, the most accurate wave-length measurements in 1913 were those of Moseley, with an accuracy of about 1 %. By a brilliant succession of improvements in methods, design, inventions and technique, Siegbahn by 1924 had improved this to 0·001 %, a factor of 1000.

Siegbahn is not only a great physicist, he is also a great engineer. He has made inventions and improvements in almost every useful type of apparatus connected with X-ray measurements, pumps, gratings, X-ray tubes, ruling machines, etc.

Among his many achievements are the determination of the structure of the L series of X-ray spectra, the discovery of the anomalous dispersion of X-rays (with Hjalmar), the accurate and direct measurement of the grating spaces of calcite and rock salt, and the selection rules for the frequencies of X-ray absorption edges. In conjunction with Larsson and Waller, he was the first to deviate X-rays with a prism. They developed this method till they could obtain by it measurements of refractive indices for X-rays which are of quite surprising accuracy.

In recent years he has been much occupied with the development and improvement of methods of measurement of 'ultra-soft' X-rays, the region between about 10 to 500 A, and with conspicuous success. This covers the gap between the ordinary X-ray region and the optical region of radiation.

He and his students have also been much interested in the values of the fundamental constants of physics and have made important contributions to our knowledge of them.

He has written a masterly book on the spectroscopy of X-rays. An extraordinarily large proportion of the information in it is due to the work of himself and his students. He has created an outstanding school at Uppsala and Stockholm, which now represents most of the physics of Sweden.

He was awarded the Hughes Medal in 1934.

A ROYAL MEDAL is awarded to Professor PATRICK MAYNARD STUART BLACKETT.

Professor Blackett is especially distinguished for his work on cosmic rays and the particles connected with them.

The early work which first brought his name into prominence was concerned with the disintegration of nitrogen by α-particles; arising out of the experimental observations was the convincing proof that the disintegration process originated in the actual capture of the α-particle by the nitrogen nucleus.

The demonstration of cosmic-ray showers was one of Blackett's early successes with direct Wilson chamber photographs, but perhaps his most spectacular discovery—made simultaneously by Anderson in America—was that, in a large cloud chamber controlled by the tripping of counters, tracks appeared which could only be explained as due to a new particle—

the positive electron. The importance of this discovery in the light of Dirac's theory was immediately realized by Blackett and his co-worker Occhialini, and important results have emerged.

Blackett also—in collaboration with Chadwick and Occhialini—extended the work on the positive electron, and it was soon found that there were sources other than cosmic rays. With the same collaboration Blackett was also instrumental in showing that quanta of sufficient energy could produce a pair of electrons and this production was related to the so-called nuclear absorption of γ-rays—a phenomenon previously known but until then unexplained.

Blackett has followed up his cosmic-ray work and has published a number of very interesting papers dealing with various aspects of these rays. He has measured their energy, inferring therefrom a cosmic-ray energy spectrum; he has observed, with an extraordinarily high degree of accuracy, the scattering and energy loss of cosmic-ray particles in their passage through metal plates and has discussed the nature of the penetrating component of Cosmic Rays. Two papers on this topic appeared in the *Proceedings of the Royal Society* for 1938.

Lastly, reference must not be omitted of Blackett's important experimental contributions to our knowledge of the heavy electron—the particle which seems destined to be of such importance in the understanding of the more familiar nuclear particles.

A ROYAL MEDAL is awarded to DR FRANCIS HUGH ADAM MARSHALL.

Dr Marshall's earlier research work (1903–1907) on the oestrous cycle, corpus luteum, and removal and grafting of ovaries laid the foundations for all the modern discoveries concerning the internal secretions of the sex organs.

The publication of his large text-book on the *Physiology of Reproduction*, in 1910, stimulated work on this subject throughout the world, not only on points of scientific interest but also in regard to the application to medicine and to questions of fertility and milk secretion in the domestic animals. He was engaged on a third edition of this book when war broke out. He is generally acknowledged to be the father of this subject, and but few papers on this branch of science to-day are published without some reference to his work.

Arising out of his research, and that of his pupils, the importance of the anterior pituitary as a source of internal secretions, affecting not only the sex organs but also other body functions, has been recognized, and has led the way to an enormous volume of research work in recent years throughout the world. It is one of the subjects in which the greatest advances have been made in recent years.

Recently, his research has been concentrated on the exteroceptive factors, such as light, ultra-violet irradiation and nerve stimuli, which affect the sex organs by way of their effects on the anterior pituitary. This work explains the physiological basis for the seasonal and other changes which occur in reproductive activity. He summarized this aspect of the subject in the 1936 Croonian Lecture on 'Sexual periodicity and the causes which determined it', beside adding new matter.

The DAVY MEDAL is awarded to Professor HAROLD CLAYTON UREY.

Professor Urey's first important piece of work consisted in carrying out extensive, accurate, spectroscopic measurements on diatomic and polyatomic molecules. This led him in 1931 to take up a detailed investigation on the abundance of natural isotopes of hydrogen, nitrogen and oxygen. During the next few years he succeeded in isolating deuterium and calculating the comparative thermodynamic properties of deuterium, hydrodeuterium and hydrogen. In 1934 he accomplished the first synthesis of deuteromethane.

Deuterium or 'labelled' hydrogen has proved of great value in investigating the mechanism of many organic and biologically important reactions, and its use has been the precursor of the modern general isotopic exchange reactions. A number of deutero derivatives have been prepared by Urey and his co-workers, and their entropies, vapour pressures and exchange equilibrium constants have been experimentally determined and compared with the theoretical values anticipated.

This isolation of deuterium from ordinary hydrogen and establishment of the thermodynamic, spectral and physico-chemical difference between it and pure hydrogen, as well as in the compounds containing deuterium and hydrogen, is a remarkably complete piece of work, for which Urey received the Nobel Prize.

More recently Urey has taken up the problem of the separation of the other important, naturally occurring isotopes: those of nitrogen, oxygen and carbon. He has examined their quantity distribution in nature and employed exchange methods for the enrichment of one species.

The DARWIN MEDAL is awarded to Professor JAMES PETER HILL.

Over a long series of years Professor Hill has carried out researches on the development of various mammals, particularly as regards the embryonic membranes and placenta, and added greatly to our knowledge of this subject. Many of his conclusions have clear evolutionary implications, as for example that marsupials are descended from oviparous ancestors with meroblastic ova. In his Croonian Lecture of 1932 Professor Hill summarized his researches on the embryology and embryonic membranes of the Primates. The views of primate evolution based on development which he then put forward are in accord with those of Elliot Smith founded on brain anatomy, and of W. K. Gregory on morphological and palaeontological evidence.

In collaboration with T. T. Flynn, Professor Hill has lately (1939) published the first part of extensive researches on the development of monotremes, both *Ornithorhynchus* and *Echidna*, which will be of great value in helping to assess the origin and relationships of these egg-laying mammals.

Professor Hill's research work is of first-class quality, being trustworthy and carried out with extreme care and the best techniques; it has never been scamped and is rich in original results over a wide field, most of the conclusions having a direct bearing on evolutionary questions.

Few living biologists have contributed more towards the solution of problems bearing on the interrelationships of the main groups of the Mammalia and on the phylogenetic history of the Primates, a subject with which Charles Darwin was so much concerned.

The SYLVESTER MEDAL is awarded to Professor GODFREY HAROLD HARDY.

G. H. Hardy is the author, or part author, of over 300 mathematical papers, two books, and several of the Cambridge Mathematical Tracts.

Much of his work has been directed to the building up of the technique of modern mathematical analysis, and the simplicity with which the routine aspects of new work can now be presented is due very largely to fundamental results established by him.

It is characteristic of much of his work that it has stimulated others and has proved to be the starting point of important developments. His work in collaboration with J. E. Littlewood on Tauberian theorems is an example. From an isolated classical result a subject was created which to-day would require a treatise for its exposition.

His most outstanding contributions to the advance of mathematical knowledge have been in the theory of the Riemann zeta-function and the theory of numbers. The achievement of which, it is believed, he himself is most justly proud is the invention of the 'circle method'. This is a technique of great beauty and generality which brings great refinement of mathematical analysis to bear on a wide class of unsolved problems in the theory of numbers.

No appreciation of the services of Hardy to the advance of mathematics would be complete which did not attempt to assess the value of his personal influence. Throughout his career he has been the driving force behind a vigorous group of younger research workers. A very considerable proportion of the pure mathematical research now being published in this country is traceable more or less directly to his interest and encouragement, or to the inspiration of his earlier work. His unstinted service during many years to the detailed work of the London Mathematical Society, and the freedom with which his experience and advice are available to all, have established him in a unique position in the regard of British mathematicians.

The HUGHES MEDAL is awarded to Professor ARTHUR HOLLY COMPTON.

Professor Compton has made a number of important contributions to physical science in the field of X-rays and elsewhere. Of late years he has been one of the leaders in the study of cosmic rays.

The experiments of Young and Fresnel early in the nineteenth century proved that light certainly had undulatory properties. But in the present century facts have been emerging, notably in connexion with photoelectric action, which are impossible to reconcile with the assumption that light can be described only as an electromagnetic wave of the classical type. These difficulties disappeared if light of frequency ν is assumed to be dynamically equivalent to a collection of particles of energy $h\nu$ (h = Planck's constant).

It occurred to Compton that from this standpoint the interaction between radiation and free electrons is very simple, and in fact is the simplest interaction which radiation can undergo. Associated with the energy $h\nu$, according to the electromagnetic theory, there is momentum $h\nu/c$ (c = velocity of light). The interaction is thus reduced to a very ancient problem, that of the encounter of two infinitesimal billiard balls with known energies and momenta. As the radiation moves with the velocity of light, in most cases the electron can be treated as if it were at rest. It is then obvious that in the collision the electron will acquire energy from the radiation and the conservation of momentum requires that if the electron moves off in a certain direction the radiation will travel in a certain other direction. But reduction of energy of a quantum of radiation means increase in wave-length, and this increase will be a predetermined function of the direction of the 'scattered' radiation and of the direction of motion of the 'recoil' electron.

He published these conclusions in 1922. In 1923 he established the change in wave-length, first qualitatively by Barkla's absorption coefficient methods and then quantitatively with the X-ray spectrometer. In the succeeding years he investigated the energies of the recoil electrons as a function of their direction of motion and showed that the correlation, predicted by the theory, between the direction and energy of the recoil electrons on the one hand and the direction and change of wave-length of the radiation on the other did in fact occur. This correlation is of fundamental importance in the general theory of the interaction of radiation with matter.

The production of cancer by pure hydrocarbons. V

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Tests for carcinogenic activity have been carried out with about seventy compounds, mostly new substances specially synthesized for the purpose and related to known carcinogenic compounds. The results support the view that there is a definite association between molecular structure and carcinogenic action; thus in the 3:4-benzphenanthrene series, which has not hitherto been investigated extensively, positions 1 and 2 seem to be the favourable points for substitution. The capacity to produce epithelioma does not always run parallel with the capacity to produce sarcoma. Some preliminary observations are given upon the occurrence of multiple tumours in animals receiving some classes of these compounds; this subject requires much further investigation with animals in which the spontaneous incidence of such tumours is known.

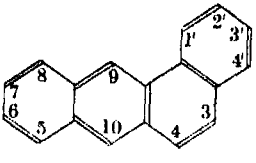
The results of tests for cancer-producing activity carried out with numerous compounds, mostly polycyclic aromatic hydrocarbons, have been described in four earlier communications (Cook, Hieger, Kennaway and Mayneord 1932; Cook 1932; Barry, Cook, Haslewood, Hewett, Hieger and Kennaway 1935; Bachmann, Cook, Dansi, de Worms, Haslewood, Hewett and Robinson 1937). The test procedure employed consisted chiefly in the production of epitheliomas by application to the skin of mice of dilute solutions of the compound in benzene or other solvent. Some of the active compounds were also injected, dissolved in a fatty medium, into the subcutaneous tissues of mice or rats, and were found to give transplantable tumours having the character of sarcomas. This type of technique, introduced by Burrows, Hieger and Kennaway (1932), has been much extended by Shear (1936, 1938, 1939; Fieser, Fieser, Hershberg, Newman, Seligman and Shear 1937) who has adopted as a standard test procedure the implantation of the compound, either in the crystalline state or in cholesterol pellets, into the subcutaneous tissues of mice. In general there was good correspondence between the orders of carcinogenic potencies of compounds which were assayed both by application to the skin and by injection. However, comparison of the activities of simple homologues of 1:2-benzanthracene revealed that

10-methyl-1:2-benzanthracene showed much greater carcinogenic potency in tests carried out by Shear by implantation than when the compound was applied to the skin in this Institute. Conversely, 9:10-dimethyl-1:2-benzanthracene, which produced tumours in a surprisingly short time on application to the skin of mice (Bachmann, Kennaway and Kennaway 1938), was found by Shear (1938) to give tumours much more slowly and in a smaller proportion of the mice when tested by his technique. This lower activity in producing sarcomas has been confirmed with the mice used in this Institute, the hydrocarbon being injected in solution in sesame oil. Hence, in tests for carcinogenic activity carried out with a new series of compounds now reported, in a large proportion of cases the compound was tested by application to the skin of mice, usually in 0·3 % solution in benzene, and also by injection *sub cutem*, usually in sesame oil (usual dose 2·5 or 5 mg. in 0·2 c.c. of oil); the injection was repeated at intervals of three to five weeks according to the rate of disappearance of the quantity given previously. The results have shown again that some compounds are more effective in producing epitheliomas than sarcomas, while with other compounds the reverse is true.

For the most part the compounds dealt with are related to the known groups of carcinogenic hydrocarbons, and detailed discussion of the results summarized in tables 1–6 is unnecessary. Methods used for the synthesis of the compounds have been described in a number of papers published in the *Journal of the Chemical Society* (Badger, Cook, Goulden, Hewett, Robinson and de Worms 1937–40). New data with compounds specifically synthesized for biological test have enabled further generalizations to be made regarding the influence of changes in molecular structure on carcinogenic activity, and additional emphasis is given to the conclusion that certain types of molecular structure are inherently associated with carcinogenic properties. It is of considerable interest that some of the active compounds are derivatives of the hydrocarbons in which substituent groups contain nitrogen or oxygen, a fact which has also been elucidated in the results reported by Shear (1938, 1939) with compounds synthesized by Fieser and his collaborators. Such findings may ultimately be of importance in explaining the role of compounds in the process of carcinogenesis.

COMPOUNDS DERIVED FROM 1:2-BENZANTHRACENE (TABLES 1 AND 2)

Although the parent hydrocarbon 1:2-benzanthracene has very little carcinogenic activity it may be regarded as a potential carcinogen, and it is now clear that activity is developed when suitable substituents are introduced into one or more of several of the twelve positions of substitution in the molecule.



1:2-Benzanthracene.

Extensive tests have now been made with the monomethyl derivatives of 1:2-benzanthracene, all of the twelve possible isomerides having been synthesized and examined. The only compounds in this series which have given tumours in a large proportion of the mice, both by application to the skin and by injection, are the 5-, 9-, and 10-methylbenzanthracenes. Other methylbenzanthracenes (substituents at positions 3, 4, 6, 7, 8, 4′) have given occasional skin tumours. Two of the compounds in question (3- and 7-methylbenzanthracenes) had been reported as inactive in earlier tests, but have now given a few epithelial tumours in experiments of longer duration carried out with larger numbers of mice. These two compounds are, however, very effective in producing sarcomas by subcutaneous injection. 7-Methyl-1:2-benzanthracene gave sarcomas in four out of twenty mice, and 3-methyl-1:2-benzanthracene in ten out of twenty mice. The remaining three methylbenzanthracenes (substituents at positions 1′, 2′ and 3′) have not given tumours.

The experiments of Haddow and Robinson (1937, 1939) on the correlation between growth-inhibitory action and carcinogenic activity of polycyclic compounds had shown that the 3- and 7-methyl derivatives of 1:2-benzanthracene were anomalous inasmuch as they produced strong inhibition of growth, although our earlier tests had not given tumours when the compounds were applied to the skin of mice. The experiments now reported on the marked sarcoma-producing power of these two hydrocarbons remove this discrepancy. Similar considerations apply in the case of 'acenaphthanthracene'; this hydrocarbon, which also gave negative results in earlier tests for carcinogenic action, showed marked inhibition of tumour growth. In more extensive tests the compound has given ten tumours (five epitheliomas and five papillomas) on application to the skin of fifty mice, and has also given sarcomas in six out of ten mice into which it was injected subcutaneously. Several of the benzanthracene derivatives dealt with in the present communication have also been tested by Shear (1938,

TABLES 1–6. ABBREVIATIONS

Methods of administration: A = application to skin. B = injection *sub cutem*. C = by the mouth. s. of i. = site of injection. If no other statement is made, the method of administration was (1) by application to the skin twice weekly in benzene (chiefly 0·3 % solution); (2) by injection *sub cutem* (5 mg. in 0·2 c.c. sesame oil) at intervals of a few weeks; and (3) by the mouth, either mixed with the food in 2 or 4 % solution in olive oil, or by stomach tube (1 mg. in 0·25 c.c. butter, or latterly margarine, to each mouse on 5 days weekly). Pap. = papilloma. Epithma. = epithelioma. Sebac. adenom. = sebaceous adenoma. Numbers in brackets show earliest and latest days of death of tumour-bearing mice, and these bracketed numbers are used also in last column to denote individual mice or tumours, which came to autopsy on the days stated.

TABLE 1. DERIVATIVES OF 1:2-BENZANTHRACENE

Compound, and method of administration		Initial no. of mice	Day of death of last mouse	No. of mice bearing tumours: Skin: Papilloma	No. of mice bearing tumours: Skin: Epithelioma	Sarcoma	Details of tumours. Other tumours. Metastases
3-Methyl-	A	92	707	2 (520, 707)	2 (508, 693)	—	Mouse bearing epithma. (508) had also sebac. adenom. on abdomen. Pap. (520) was cystic sebac. adenom.
	B	20	276	—	—	10 (143, 253)	
7-Methyl-	A	30	687	2 (563, 618)	0	—	Mouse (563) had also sebac. adenom. on eyelid
	B	20	309	—	—	4 (307, 363)	
8-Methyl-	A	20	682	1 (343)	1 (682)	—	
1′-Methyl-	A	20	410	1 ? (351)	—	—	
	B	10	628	—	—	0	
4′-Methyl-	A	40	622	0	1 (622)	—	

9-Methyl-	A	40	528	3 (268, 407)	3 (313, 528)	—	Two paps. (268 and 310) were accompanied by peculiar cystic pap. Mouse bearing epithma. (313) had also lympho-sarcoma invading lung
10-Methyl-	A	70	280	3 (102, 281)	20 (138, 270)	—	Metastasis in lung (270)
Acenaphthanthracene (3:4-dimethylene-1:2-benzanthracene)	A	50	535	5 (413, 535)	5 (277, 493)	—	
	B	10	298	—	—	6 (181, 280)	
5-*iso*-Propyl-	A	20	338	1 (337)	6 (248, 321)	—	
	B	10	378	—	—	3 (314, 378)	
5-*n*-Butyl-	A	20	406	1 (406)	8 (250, 408)	—	The epitheliomas tend to be multiple, and of rodent ulcer type, and to be accompanied both by papillomas also of rodent ulcer type and by sebac. adenomas. Five out of eight epithma.-bearing mice show these characters
	B	10	591	—	—	0	
5-*n*-Amyl-	A	20	454	3 (233, 435)	7 (277, 448)	—	Five out of seven epithma.-bearing mice show, though to less degree, characters of 5-*n*-butyl series
	B	10	460	—	—	0	
5-*n*-Hexyl-	A	20	471	4 (451, 472)	2 (310, 353)	—	Two of the paps. are accompanied by sebac. adenoma. One epithma.-bearing mouse has sebac. adenom. on leg
5-*n*-Heptyl-	B	10	542	—	—	0	
5-Ethyl-7:8-dihydro-	A	10	644	0	0	—	
5-Phenyl-	A	10	485	0	1 (483)	—	
9:10-Dimethyl-	A	20	172	5 (83, 123)	8 (83, 172)	—	
	B	40	165	—	—	3 (106, 165)	One mouse had epithma. over s. of i., no sarcoma
9:10-Diethyl-	A	20	424	4 (113, 425)	0	—	One mouse had several paps., some of sebac. adenom. type

TABLE 1 (*continued*)

Compound, and method of administration		Initial no. of mice	Day of death of last mouse	No. of mice bearing tumours			Details of tumours. Other tumours. Metastases
				Skin			
				Papilloma	Epithelioma	Sarcoma	
5:9:10-Trimethyl-:							
0·06 % in benzene	A	26	193	7 (69, 137)	10 (89, 193)	—	One mouse had epithma. and melanoma. at s. of i. and epithma. on jaw; another had pap. and melanoma at s. of i.
0·3 % in benzene	A	40	119	6 (60, 124)	0	—	One mouse had pap. and melanoma at s. of i.
	B	10	231	—	—	4 (154, 231)	
6:9:10-Trimethyl-	A	20	171	3 (114, 172)	8 (91, 171)	—	Nearly all mice showed multiple, e.g., a dozen, paps. on back
	B	10	320	—	—	2 (121, 319)	
5:6:9:10-Tetramethyl-	A	20	202	1 (100)	8 (168, 202)	—	Several mice bore multiple epithmas.: (1) on back and foreleg; (2) same; (3) on back and forehead; (4) on back, thorax and eyelid; or epithma. with paps.
dose 2 mg.	B	10	212	—	—	1 (157)	
10-Hydroxymethyl-:							
0·3 % in acetone	A	10	289	1 (287)	7 (204, 285)	—	One mouse bore epithma. on head and shoulder
	B	10	238	—	—	10 (115, 239)	(1) Sebac. adenom. involving rectum. (2) Metastasis in lymphg land by right ureter
10-Acetoxymethyl-	A	20	321	3 (283, 321)	8 (218, 321)	—	Three mice bore paps. and sebac. adenom.; and another a malignant sebac. tumour
	B	10	126	—	—	8 (118, 126)	

10-Ethoxymethyl-	A	20	481	4 (350, 481)	2 (379, 447)	—	Paps. and epithmas. in part of peculiar small-celled type
	B	10	292	—	—	0	Sebac. adenom. on foreleg
10-β-Hydroxyethyl-	B	10	279	—	—	3 (179, 269)	
9:10-Bishydroxymethyl-	B	10	535	—	—	3 (283, 357)	
9:10-Bisacetoxymethyl-	A	10	555	1 (498)	0	—	Mouse with pap. (498) had peculiar wide pap. in stomach
	B	10	454	—	—	3 (318, 419)	
9:10-Dimethyl-1:2-benzanthracene-9:10-oxide	A	20	353	3 (265, 349)	5 (253, 353)	—	One mouse bore on back epithma. and several paps. of rodent ulcer and other types
9:10-Dimethyl-1:2-benzanthracene-9:10-peroxide	B	10	346	—	—	0	
10-Amino-	B	10	230	—	—	2 (159, 159) and 2? malig.	
10-Cyano-	B	10	333	—	—	4 (249, 333)	Lymphosarcoma invading both kidneys
9-Methyl-10-cyano-	A	10 C_3H	180	2	6	—	
	B	10 C_3H	169	—	—	7	
Sodium 1:2-benz-5-anthroate:							
0·3 % in alcohol	A	30	612	0	0	—	Epithma. of neck
10 *sub cutem*, 10 peritoneum, 0·3 % in water	B	20	551	—	—	0	
Sodium 1:2-benzanthranyl-5-acetate: 2 % in water, 4 doses only of 10 mg.	B	10	296	—	—	0	
9:10-Dihydro-1:2:5:6-dibenzanthracene (Bachmann method)	A	10	619	2 (337, 526)	0	—	One pap. (337) with sebac. adenom.

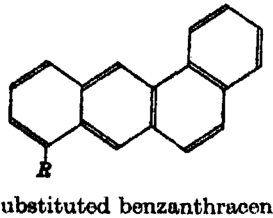
TABLE 2. DERIVATIVES OF CHOLANTHRENE AND 3:4-BENZPYRENE

Compound, and method of administration		Initial no. of mice	Day of death of last mouse	No. of mice bearing tumours			Details of tumours. Other tumours. Metastases
				Skin			
				Papilloma	Epithelioma	Sarcoma	
20-Methylcholanthren-15-one, 0·1 % in acetone	A	20	367	0	4 (292, 355)	—	No tumours of lung or stomach in eleven mice examined between 259th and 370th day
3-Hydroxy-20-methylcholanthrene, 0·3 % in acetone	A	10	549	0	0	—	
3-Methoxy-20-methylcholanthrene	A	10	406	0	0	—	
4′-Hydroxy-3:4-benzpyrene, 0·3 % in acetone	A	10	434	0	0	—	
4′-Methoxy-3:4-benzpyrene	A	10	444	0	0	—	
3:4:8:9-Dibenzpyrene	A	30	350	0	10 (202, 354)	—	Metas. in lung in two mice bearing epithma. (291, 298)
3(N)-4-Pyridinopyrene	A	20	748 (one still alive)	0	0	—	
Anthanthrene	A	30	712	0	0	—	Very large adenoma of lung (615)
Pyrene	A	40	680	0	0	—	

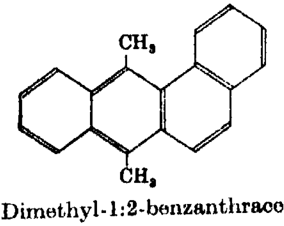
1939). For the most part the samples used by him were prepared by different synthetic methods from those by which our own specimens were obtained, and he used pure strain mice whereas we have used stock mice for the most part. There is very good agreement between the results obtained in the two laboratories.

The pronounced carcinogenic activity of the 5-, 9-, and 10-methylbenzanthracenes indicated that it would be of interest to determine the influence of other alkyl groups in these positions, and we have taken up this question in the case of the 5-substituted compounds. Tests have shown that epithelial tumours are produced in mice with the 5-ethyl-, 5-*n*-propyl-, 5-*n*-butyl-, 5-*n*-amyl-, 5-*n*-hexyl-, 5-*iso*propyl-, and 5-phenyl-derivatives of 1:2-benzanthracene. Tumours were not obtained with 5-*n*-heptyl-1:2-benzanthracene which is still under test by application to the skin.*

It is evident from these results that substitution at position 5 of the benzanthracene molecule gives pre-eminently a favourable structure for carcinogenic activity. Sarcomas have been obtained with 5-*iso*propyl-1:2-benzanthracene in three out of ten mice, whereas the 5-*n*-butyl, 5-*n*-amyl and 5-*n*-heptyl compounds have not given tumours by this method. A series of 10-alkyl derivatives of 1:2-benzanthracene has been synthesised by Fieser and Hershberg (1937), but results of biological tests have not yet been reported. However, the complete inactivity of 10-*iso*propyl-1:2-benzanthracene (Barry *et al.* 1935) suggests that the carcinogenic activity of 10-methyl-1:2-benzanthracene is much more susceptible to changes in molecular structure than is that of 5-methyl-1:2-benzanthracene.



5-Substituted benzanthracenes.



9:10-Dimethyl-1:2-benzanthracene.

Until recently the most rapidly acting carcinogenic compound was 20-methylcholanthrene, which gives in mice epithelial tumours which appear some 75 days after the beginning of application. A much more rapidly acting compound was found in 9:10-dimethyl-1:2-benzanthracene which gave tumours in less than half this time (Bachmann *et al.* 1938). Homologues of 9:10-dimethyl-1:2-benzanthracene have been synthesized and tested and also show a high order of activity in the production of skin tumours. Like 9:10-dimethyl-1:2-benzanthracene they are less effective in producing sarcomas. Skin tumours were obtained after a short induction period with 5:9:10-, and 6:9:10-trimethylbenzanthracenes. With some of the earlier compounds tested in this Institute it was found that the optimum concentration for application to the skin was about 0·3%. In the case of 5:9:10-trimethylbenzanthracene this concentration was found much less effective than 0·06%, a circumstance which is perhaps associated with the high potency of this compound. Tumours were obtained less readily with 9:10-diethyl-1:2-benzanthracene, which gave only non-malignant tumours of the skin; 5:6:9:10-tetramethyl-1:2-benzanthracene, although active in the production of skin tumours, was markedly slower in action than the trimethyl compounds.

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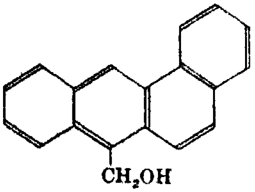
* (*Note added at correction of proof.*) One epithelioma has been obtained in 574 days from a series of 20 mice.

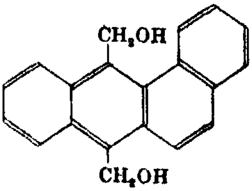
Subcutaneous injections were made of the 9:10-peroxide formed by atmospheric oxidation of 9:10-dimethyl-1:2-benzanthracene in the presence of light. On account of the instability of the compound and the necessity for preparing new specimens for each injection the treatment was not maintained over a long period. Tumours were not obtained, and it seems unlikely that such a peroxide is an intermediate product when the hydrocarbon acts as a carcinogenic agent. However, the 9:10-oxide of 9:10-dimethyl-1:2-benzanthracene gave skin tumours in eight out of twenty mice.

Fieser and his collaborators have reported the production of sarcomas by subcutaneous injection in mice of several benzanthracene derivatives containing substituents such as hydroxy, methoxy, carbomethoxy, amino and cyano. For instance, Shear obtained tumours with 10-hydroxymethyl-1:2-benzanthracene (reported by Fieser and Hershberg 1938). In our hands also this compound proved very efficacious in producing sarcomas (tumours were obtained in all ten of the injected mice), and it also gave epitheliomas in seven out of ten mice to which it was applied in acetone. Skin tumours were also obtained with the acetate and (less readily) the ethyl ether of this carbinol; the latter derivative gave no sarcomas, the former gave sarcomas in eight out of ten mice.

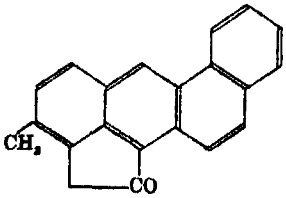
The effect of substitution in the 9- and 10- positions of 1:2-benzanthracene requires further investigation. Thus the 10-amino and 10-cyano derivatives have given at the time of writing no tumours of the skin of stock mice in 275 and 387 days, although both have given sarcomas in shorter times; while the 9-methyl-10-cyano- compound gave (in C_3H mice) two papillomas and six epitheliomas in 180 days (table 1). The part played here by the strain of mice cannot yet be stated; the C_3H mice are known to produce sarcomas rapidly (Andervont 1938) and ten of these mice receiving injections of the 9-methyl-10-cyano- compound have given seven sarcomas in 169 days, but their capacity to produce epitheliomas has not yet been compared with that of stock mice.



10-Hydroxymethyl-1:2-benzanthracene.



9:10-Bishydroxymethyl-1:2-benzanthracene.



20-Methylcholanthren-15-one.

Oxygen derivatives of the highly carcinogenic 9:10-dimethyl-1:2-benzanthracene have also given tumours. 9:10-Bishydroxymethyl-1:2-benzanthracene and its diacetate both gave sarcomas. The latter compound, when tested by application to the skin, gave one non-malignant tumour. The production of skin tumours by oxygen derivatives of 10-methyl-1:2-benzanthracene is of considerable interest, and it is also noteworthy that epitheliomas were obtained with 20-methylcholanthren-15-one, a compound shown by Shear (1939) to give tumours at the site of injection. Our sample of this ketone was isolated from the products of oxidation of methylcholanthrene with chromic acid in cold acetic acid. It was sublimed in a high vacuum and repeatedly crystallized from benzene, and had m.p. 256–257°. (Found: C, 89·1; H, 5·0. Calc. for $C_{21}H_{14}O$: C, 89·1; H, 5·0 %.) (Compare Fieser and Hershberg 1938.)

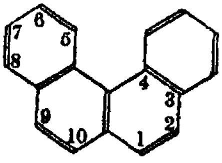
In Part III reference was made to the production of two epitheliomas in ten mice by application of 9:10-dihydro-1:2:5:6-dibenzanthracene. It was subsequently found that the sample used was heavily contaminated with 1:2:5:6-dibenzanthracene. A new experiment carried out with the pure dihydride has given only two papillomas.

3(N)-4-Pyridinopyrene (Vollmann, Becker, Corell and Streeck 1937) has given no tumours of the skin in twenty mice in 748 days and no sarcomas in 182 days (one mouse still alive in each series).

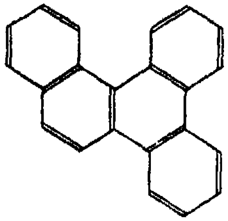
COMPOUNDS RELATED TO 3:4-BENZPHENANTHRENE (TABLE 3)

The production of epitheliomas after a prolonged latent period by 3:4-benzphenanthrene and the much greater activity of its 2-methyl derivative have been described already (Parts III and IV), and further attention has been devoted to compounds having similar types of molecular structure. As in the benzanthracene series an endeavour has been made to survey the field by examining all of the possible monomethyl derivatives and also preparing a series of higher homologues with selected positions of substitution. In addition, tests have been carried out with three newly synthesized pentacyclic hydrocarbons built up of benzene rings, all three being related to 3:4-benzphenanthrene. These compounds are 1:2:3:4-dibenzphenanthrene, 1:2:5:6-dibenzphenanthrene, and 1:2-(1′:2′-naphtha)-anthracene. The last named compound is also related to 1:2-benzanthracene.

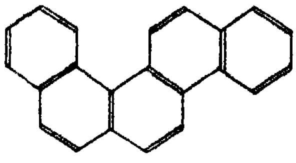
In many cases tests have been in progress for a short time only so that a final discussion of the results cannot be attempted.



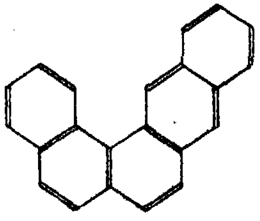
3:4-Benzphenanthrene.



1:2:3:4-Dibenzphenanthrene.



1:2:5:6-Dibenzphenanthrene.

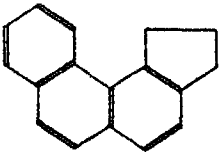


1:2-(1′:2′-Naphtha)-anthracene.

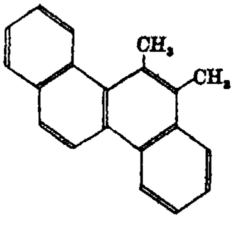
The 3:4-benzphenanthrene molecule is more symmetrical than that of 1:2-benzanthracene and only six monomethyl derivatives are possible. With the exception of the 5-methyl compound these have all been synthesised. All five monomethyl benzphenanthrenes have shown indications of carcinogenic activity when applied to the skin of mice, although with the 6-methyl compound only one papilloma in twenty mice was obtained. When tested by subcutaneous injection only the 2-methyl and the 6-methyl compounds showed slight activity, the remaining three compounds giving no tumours. On the whole, the results suggest that the group of 3:4-benzphenanthrene derivatives is more active in producing epitheliomas than in producing sarcomas.*

The marked activity of 2-methyl-3:4-benzphenanthrene is shown also by the 2-*iso*propyl compound which gave fourteen tumours (six epitheliomas and eight papillomas) in a series of twenty mice. Tests are in progress with the 2-ethyl and 2-*n*-propyl compounds and also with 1-*iso*propyl-3:4-benzphenanthrene. It is noteworthy that the carcinogenic activity of the parent hydrocarbon, 3:4-benzphenanthrene, has been confirmed with a specimen prepared by a completely different synthetic method from that originally used.

The results suggest that position 2 of the benzphenanthrene molecule is the position of substitution most favourable for carcinogenic activity and that position 6 is unfavourable. This is borne out by tests of the pentacyclic hydrocarbons. 1:2:3:4-Dibenzphenanthrene (additional ring attached at positions 1 and 2) has proved very efficacious in producing both epitheliomas and sarcomas; 1:2:5:6-dibenzphenanthrene (additional ring at positions 7 and 8) showed much less activity by both test procedures; 1:2-(1′:2′-naphtha)-anthracene (additional ring at positions 6 and 7) gave no skin tumours in an experiment with ten mice lasting for 544 days. Contraction of one of the benzene rings of the 3:4-benzphenanthrene molecule appears to destroy the carcinogenic activity, for no tumours were obtained in a painting experiment with 3:4-*cyclo*pentenophenanthrene.



3:4-*cyclo*Pentenophenanthrene.



1:2-Dimethylchrysene.

In view of the relationships established in the benzanthracene series where from the standpoint of carcinogenic activity two methyl groups

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* Newman and Joshel (1940) have reported that the 2:9-dimethyl, 2:9-diethyl, 6:7-dimethyl and 2-*iso*propyl-8-methyl derivatives of 3:4-benzphenanthrene are all inactive when tested by injection.

TABLE 3. COMPOUNDS RELATED TO 3:4-BENZPHENANTHRENE

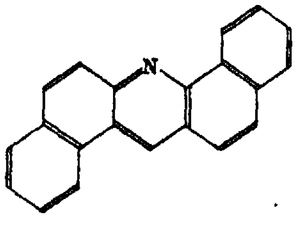
Compound, and method of administration		Initial no. of mice	Day of death of last mouse	No. of mice bearing tumours			Details of tumours. Other tumours. Metastases
				Skin			
				Papilloma	Epithelioma	Sarcoma	
3:4-Benzphenanthrene	A	40	580	1 (548)	4 (355, 594)	—	
	B	10 mice	445	—	—	0	
		6 rats	533	—	—	1 (533)	
1-Methyl-	A	20	335	2 (213, 313)	6 (226, 335)	—	
	B	10	329	—	—	0	
	C	10	527	—	—	—	
2-Methyl-, Part IV	A	20	300	5	7	—	
	B	20	459	—	Two had epithmas. over s. of i.	3 (186, 354)	One grafted sarcoma died out in 3rd grafted generation. Two mice had epithma. over s. of i. Others had (1) epithma. on back and on abdomen and pap. on chest, (2) large pap. at side of mouth. On multiple tumours in mice 4339 and 4173, see text, p. 460
	C	10	383	—	—	—	Epithma. and sebac. adenom. of neck (356)
2-Ethyl-	A	10	272	3 (200, 250)	2 (214, 228)	—	
2-*iso*-Propyl-	A	20	419	8 (213, 419)	6 (283, 419)	—	
	B	10	482	—	—	0	Extensive sarcoma of abdom. cavity; squamous pap. of stomach; nil s. of i. (482)
	C	10	448	—	—	—	
6-Methyl-	A	20	678	1 (617)	0	—	
	B	10	674	—	—	2 (466, 519)	Sarcoma, and many paps. all over body (519). Small round-celled tumour of testis (618)
	C	10	637	—	—	—	Carcinoma small intestine and pap. stomach (394)

7-Methyl-	A	20	641	1 (542)	2 (537, 641)	—	Pap. and sebac. adenom. (542). Epithma. partly rodent ulcer type; multiple paps. (about 16) all over skin; lymphosarcoma (641)
	B	10	624	—	—	0	
	C	10	620	—	—	—	
8-Methyl-	A	20	584	2 (430, 569)	2 (561, 584)	—	Mouse bearing interscapular pap. (569) has pap. on forehead also
	B	10	482	—	—	0	Sebac. adenom., and 2 paps. in stomach (478)
	C	10	498	—	—	—	Sebac. adenom., unusual type, on abdomen (498)
3:4-*cyclo*Pentenophenanthrene	A	10	416	0	0	—	
1:2:3:4-Dibenzphenanthrene	A	20	258	5 (212, 258)	8 (211, 258)	—	Metastasis left axilla (258). Metastasis lung (258)
	B	30	201	—	—	5 (144, 185) and one?	One sarcoma-bearing mouse (174) has squamous-cd. carcinoma of stomach, and one bearing spindle-cd. tumour of doubtful malignancy has squamous-cd. pap. of stomach
	C	10	622	—	—	—	One mouse (519) showed pap. and sebac. adenom. at base of tail; epithma. at side of neck; adenoma of lung and columnar-cd. tumour of lung and mediastinum ? arising from a lung adenom., and two small patches of cholangioma
1:2:5:6-Dibenzphenanthrene	A	20	577	1 (478)	1 (578)	—	
	B	15	513	—	—	2 (251, 392)	
	C	10	529	—	—	—	
1:2-(1′:2′-Naphtha)anthracene	A	10	544	0	0	—	
Sodium 6-Methyl-3:4-benz-10-phenanthroate in dist. H_2O	B	10	479	—	—	0	
1:2-Dimethylchrysene	A	20	389	1 (305)	6 (230, 389)	—	A mouse bearing one epithma. and two paps. had sebac. adenom. in groin
	B	10	469	—	—	0	Three large nodules keratinizing epithma. of lung (335)

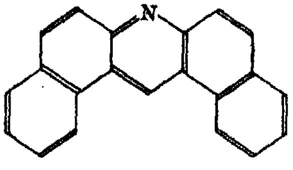
seem to be equivalent to a benzene ring attached at the same positions, it would be of interest to examine the three compounds in which the outer benzene rings of the molecule of 1:2:3:4-dibenzphenanthrene are successively replaced by pairs of methyl groups. These three compounds are (*a*) 1:2-dimethyl-3:4-benzphenanthrene, which has not yet been synthesized; (*b*) 1:2-dimethyltriphenylene which has been synthesized by Fieser and Joshel (1939), and is presumably under test; (*c*) 1:2-dimethylchrysene which has given seven tumours (six epitheliomas and one papilloma) when applied to a series of twenty mice. This is the first simple derivative of chrysene shown to have pronounced carcinogenic activity in the pure state.

OTHER COMPOUNDS (TABLES 4–6)

The tables include references to compounds other than simple derivatives of 1:2-benzanthracene and 3:4-benzphenanthrene, and for the most part these do not call for special comment. In some cases preliminary reports of tests were given in previous communications. It will be noted that tumours have not been obtained with any of the dihydrodibenzacridine derivatives. Two dibenzacridines, shown already to produce skin tumours (Barry *et al.* 1935), have now been tested by subcutaneous injection, when 1:2:5:6-dibenzacridine proved more effective than 3:4:5:6-dibenzacridine, which gave two sarcomas in ten mice. When applied to the skin the 3:4:5:6- compound seemed rather more active than the 1:2:5:6- compound.



1:2:5:6-Dibenzacridine.



3:4:5:6-Dibenzacridine.

The production of tumours of the skin in mice with 1:2:5:6-dibenzfluorene has been described already (two papillomas and six epitheliomas in forty mice in 472 days (Bachmann *et al.* 1937)). This hydrocarbon gave negative results when injected *sub cutem* and this was also the case with 1:2:3:4-dibenzfluorene and 1:2:7:8-dibenzfluorene. However, both the 1:2:3:4- and the 1:2:7:8-dibenzfluorenes gave tumours when applied to

TABLE 4. ACRIDINE DERIVATIVES

Compound; and method of administration		Initial no. of mice	Day of death of last mouse	No. of mice bearing tumours			Details of tumours. Other tumours. Metastases
				Skin			
				Papilloma	Epithelioma	Sarcoma	
1:2:5:6-Dibenzacridine:							
Part III	A	40	482	2	5	—	
Less Pure	B	9	234	—	—	5 (198, 226)	
Pure	B	10	246	—	—	3 (168, 221) and one?	Graft failed in 3rd generation
	C	10	627	—	—	—	Multiple sebac. adenom. and other tumours (442; see text, p. 460)
3:4:5:6-Dibenzacridine, Part III	A	40	597	2	11	—	
	B	10	583	—	—	2 (453, 524)	One graft positive
	C	10	572	0	0	0	
3:4:5:6-Dibenzacridine metho-sulphate, 3 % in saline	B	10	394	—	—	2 (394, 394)	Grafts negative
	C	10	426	0	0	0	
9:10-Dihydro-1:2:7:8-dibenz-acridine	A	20	700	0	0	—	
9:10-Dihydro-3:4:6:7-dibenz-acridine	A	30	672	0	0	—	
10-Phenyl-9:10-dihydro-3:4:5:6-dibenzacridine	A	30	804	0	0	—	

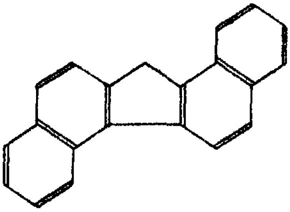
TABLE 5. FLUORENE DERIVATIVES

Compound, and method of administration		Initial no. of mice	Day of death of last mouse	No. of mice bearing tumours: Skin: Papilloma	Skin: Epithelioma	Sarcoma	Details of tumours. Other tumours. Metastases
1:2:3:4-Dibenzfluorene (none available for application to skin after 304th day)	A	20	661	One? (660) 1 (643)	1 (581)	—	Multiple paps. on back and face, some of rodent ulcer type (643)
15 mg. only in 55 days	B	10	457	—	—	0	
1:2:5:6-Dibenzfluorene, Part IV	A	40	472	2	6	—	Epithma. of lung (158)
	B	25	266	—	—	0	
1:2:7:8-Dibenzfluorene	A	20	508	2 (508, 508) sebac. adenom. (497)	3 (394, 508)	—	Two paps. one of sebac. adenom. type (508). Epithma. and paps. partly of rodent ulcer type, and sebac. adenom.
	B	10	516	—	—	0	
1′:2′-Naphtha-2:3-fluorene	A	5	885	0	0	—	

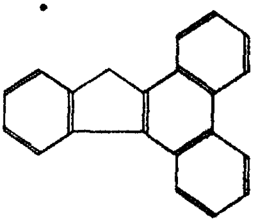
TABLE 6. OTHER COMPOUNDS

Compound, and method of administration		Initial no. of mice	Day of death of last mouse	No. of mice bearing tumours			Details of tumours. Other tumours. Metastases
				Skin			
				Papilloma	Epithelioma	Sarcoma	
Deoxycholic acid, 0·3 % in alcohol 10 vol., benzene 23 vol.	A	100	852	0	1 (776)	—	
	B	10 stock	422	—	—	4 (351, 421)	
		10 C$_3$H	207	—	—	7 (155, 207)	
4-*iso*Propylchrysene, Part IV (0·2 % in lard, 2 c.c.)	A	10	471	0	0	—	
	B	10 rats	828	—	—	0	
s-Triphenylbenzene	A	20	659	0	0	—	
	B	20	643	—	—	0	
Tetraphenylmethane	A	20	666	0	0	—	
	B	20	826	—	—	0	
Pentaphenylethane	A	20	665	0	0	—	
Triphenylethylene	A	10	517	0	0	—	
	B	10	523	—	—	0	Very large squamous-cd. pap. of stomach with some downgrowth

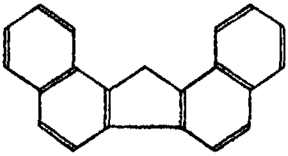
the skin, and in the latter case five tumours (three epitheliomas and two papillomas) were obtained in a series of twenty mice.*



1:2:5:6-Dibenzfluorene.



1:2:3:4-Dibenzfluorene.



1:2:7:8-Dibenzfluorene.

Thus these fluorene derivatives as well as the compounds related to 3:4-benzphenanthrene seem much more effective in producing epithelioma than in producing sarcoma.

Remarkable results, which may have far reaching implications, were obtained in tests carried out by subcutaneous injection of deoxycholic acid in sesame oil. Preliminary results have already been described (Cook, Kennaway and Kennaway 1940). This acid, which is present in combined form in bile, gave four sarcomas in ten stock mice after a prolonged latent period. In new experiments carried out with C_3H mice, sarcomas were obtained in no less than seven out of ten mice in 207 days, and one of these tumours was carried on by grafting until the 4th generation, when no further grafting was attempted. There appears thus to be no doubt that under certain conditions this compound, which is of normal occurrence in the body, is able to induce malignant tumours.

s-Triphenylbenzene and tetraphenylmethane have produced no tumours either when applied to the skin or injected *sub cutem* and this seems to be the experience of others who have tested the original statement that these two compounds are carcinogenic.*

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* The compound denoted as 1:2:7:8-dibenzfluorene is the hydrocarbon, m.p. 234°, obtained by dehydration of di-α-naphthylcarbinol with phosphoric acid as described by Tschitschibabin and Magidson (1914). The constitution assigned to this hydrocarbon is in doubt, however, for the red ketone, m.p. 269–270°, obtained by its oxidation should be identical with the 'picylenketon', m.p. 185·5°, obtained by Bamberger and Chattaway (1895) by degradation of picene. In view of this discrepancy, one of us (R. H. M.) is investigating the structure of the carcinogenic '1:2:7:8-dibenzfluorene'.

MULTIPLE TUMOURS

Multiple tumours in animals under treatment with carcinogenic compounds offer an attractive field for investigation. It is very desirable that the frequency of the spontaneous occurrence of some forms of these tumours in control animals should be established, so that the question of their value as evidence of carcinogenic action may be settled. Thus Schabad (1939) has used the occurrence of such tumours (sebaceous adenoma, adenoma of lung, carcinoma of lung and of liver, squamous carcinoma of mamma) as additional evidence of the carcinogenic action of a sarcoma-producing extract of a human liver† and of extracts of human bile, on the ground that such tumours occurred much less frequently, or not at all, in 553 control mice. At present, data, on the frequency of spontaneous tumours in various pure lines of mice are available only in the case of the mammary gland, lung, and, to some extent, the liver. The multiple tumours met with in experimental work may be classified as follows.

(1) *Multiple epithelial tumours over the region of skin to which the compound has been applied*

These are produced especially by certain substituted 1:2-benzanthracenes, namely the 9:10-dimethyl-, 5:9:10-trimethyl- (Bachmann *et al.* 1938), 6:9:10-trimethyl- and 5:6:9:10-tetramethyl compounds. The 5-*n*-butyl- and 5-*n*-amyl- compounds tend to produce multiple malignant and non-malignant tumours of diverse histological types (see table 1).

(2) *Widespread tumours of a single type, showing no special relation to the site of application of the compound*

For instance, a mouse which had received 1:2:5:6-dibenzacridine with the food during 627 days showed two epitheliomas of the skin (neck, and shoulder), epithelioma and papilloma of the stomach, numerous epitheliomas of both kidneys which had not definitely the appearance of metastases, and an epithelioma, and several small adenomata, of the lung. Hence there were at the very least three primary epitheliomas.

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* It is unfortunate that the statement that these substances are carcinogenic has now become established in the literature (Ewing 1940, p. 133).

† The production of sarcoma in mice by some extracts of human livers has been confirmed in this Institute by I. Hieger, who has obtained twelve such tumours (see forthcoming paper in the *American Journal of Cancer*).

Another mouse of the same series bore on the 442nd day about sixty small tumours scattered all over the skin; those which were examined microscopically showed the structure of cystic sebaceous adenoma. This mouse had also a squamous papilloma of the stomach and an adenoma of the lung, and hence comes within the following category also. Multiple papillomas of the skin occur also in mice receiving 6-methyl- and 7-methyl-3:4-benzphenanthrene (see table 3).

(3) *Tumours of several different tissues and organs*

For instance, a mouse (4339) which had received injections *sub cutem* of 2-methyl-3:4-benzphenanthrene showed at autopsy after 354 days a spindle-celled tumour at the site of injection, an epithelioma of the skin elsewhere, three papillomas of the stomach, and some cholangiomatous growth in the liver. Another (4173) which had received the same treatment during 248 days bore a spindle-celled sarcoma at the site of injection, a sebaceous adenoma on the chin, three small areas of cholangioma in the liver, multiple adenomata in the lungs, and two squamous papillomas in the stomach. Other instances of multiple tumours, in mice receiving 1:2:3:4-dibenzphenanthrene by injection or feeding, are given in table 3.

ADENOMA OF THE LUNG

The lungs were examined carefully with the naked eye, and every portion showing any suggestion of adenoma was excised for microscopic examination; if no such portion were found, or if the sections cut showed no adenoma, the case was recorded as negative.* The examination of the whole of both lungs by serial sections would have been impossible in view of the number of animals coming to autopsy.

The lungs of 1150 mice were treated in this way, and the results, shown in tables 7 and 8, and figures 1 and 2, indicate

(1) That only very few adenomas occur in the first 100 days of these experiments; for this reason, and in order to correct for the large number of mice examined during this period after treatment with the very active compounds 9:10-dimethyl- and 5:9:10-trimethyl-1:2-benzanthracene, the results are summarized for the two later periods, namely, from 100 to 300 days, and from 300 days onwards.

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* Some other conditions occur in the lungs of mice which differ from the usual adenomas and epitheliomas, but are apparently neoplastic; we have a number of specimens of these which are under examination.

(2) The proportion of lungs showing adenoma is lowest in the series treated with derivatives of 1:2-benzanthracene (11 %, or 12 % if the large number exposed to the 9:10-dimethyl- and 5:9:10-trimethyl- derivatives be omitted) and highest (35 %) in the series receiving the 3:4-benzphenanthrenes substituted in the 1- and 2- positions, while intermediate proportions are found in the series receiving acridines (33 %), the 6-, 7-, and 8-substituted 3:4-benzphenanthrenes (25 %), 1:2-dimethylchrysene (26 %) and the fluorenes (30 %).

TABLE 7. EPITHELIAL TUMOURS OF THE LUNG

	Days						
	100–300 tumours		300 onward tumours		Total		Percentage showing tumours
Compounds	−	+	−	+	Mice examined	Tumours +	
1:2-Benzanthracenes:							
All	213	8	129	34	384	42	11·0
Omitting 9:10-dimethyl- and 5:9:10-trimethyl-	—	—	—	—	338	41	12·1
5-*n*-Butyl-, 5-*n*-amyl-, and 5-*n*-hexyl-	—	—	—	—	55	12	21·8
3:4-Benzphenanthrenes:							
1-, 2-, 1:2:3:4-Dibenz-	49	16	19	21	105	37	35·2
6-, 7-, 8-, 1:2:5:6-Dibenz-	20	2	51	22	95	24	25·3
1:2-Dimethylchrysene	—	—	—	—	27	7	26
Acridines	0	0	14	7	—	—	33
Fluorenes	14	3	16	10	43	13	30
				Total	675	130	

TABLE 8. EPITHELIAL TUMOURS OF THE LUNG (CONTINUED)

	300th day and onward No. of mice	
	Examined	Showing epithelial tumour of lung
1:2′-Azonaphthalene, A, B, C	33	4
4′-Amino-1:2′-azonaphthalene, A, B, C	23	8
1:1′-Azonaphthalene, A, B, C	32	2
2:2′-Azonaphthalene, A, B, C	25	8
2:2′-Diamino-1:1′-dinaphthyl, A, B, C	10	2

It is noteworthy that a similar contrast between the two groups of 3:4-benzphenanthrenes appears when proliferation of the bile-ducts is under observation; this change was found in 25 % of livers exposed to the 1-, 2-group and in 4% of those exposed to the 6-, 7-, 8-group (Cook, Hewett, E. L. and N. M. Kennaway 1940). However, the 6-, 7-, 8-compounds, although they do not produce a great number of lung adenomas of the ordinary type in the first 400 days, do induce later on a remarkable number of adenomas of unusual size, and this feature does not seem to be explained by the longer life of these mice (figure 1). The great majority of adenomas of the lung in mice lie within fairly well-defined limits of size, and one can draw a useful, though of course arbitrary, distinction between these and others of larger size. The 1:2:5:6-dibenzphenanthrene appears to be especially active in producing these large adenomas. Of the 1:2-benzanthracenes, the 5-*n*-butyl-, 5-*n*-amyl- and 5-*n*-hexyl- group appears to be the most active (22% positive which is about twice the incidence in the whole 1:2-benzanthracene group) but the numbers upon which this comparison is based are of course very small.

In the five series of mice receiving the four azonaphthalenes and 2:2′-diamino-1:1′-dinaphthyl, the lungs of 264 mice were examined (table 8) and epithelial tumours were found in twenty-four (adenoma in nineteen, large adenoma in four, epithelioma in one) and all but one of these were in the group of less than one-half of these mice (123 out of 264) coming to autopsy after the 300th day; hence any action which these compounds may have in inducing such tumours is a slow one. The lowest incidence of these tumours was in the group receiving 1:1′-azonaphthalene (two in thirty-two mice).

These observations upon lung adenomas make no claim to be of more than a preliminary character; they were made upon small numbers of stock mice in which the normal incidence of these tumours at different ages is not known. Hence one does not know to what extent the increase in the frequency of lung adenomas as the experiments proceed, which is obvious in the full tables of data, is due to advancing age, or to the prolonged action of the compound. We hope that this investigation will be extended by workers who have the opportunity to apply these compounds to larger numbers of mice of various pure lines.

TUMOURS OF THE LIVER

Another part of this investigation, concerned with tumours of the liver and especially with the action of the azonaphthalenes, appears elsewhere (Cook *et al.* 1940). Here only incidental reference is made to these results, which were derived from the examination of 1004 livers from mice exposed to, in all, eighty-four compounds.

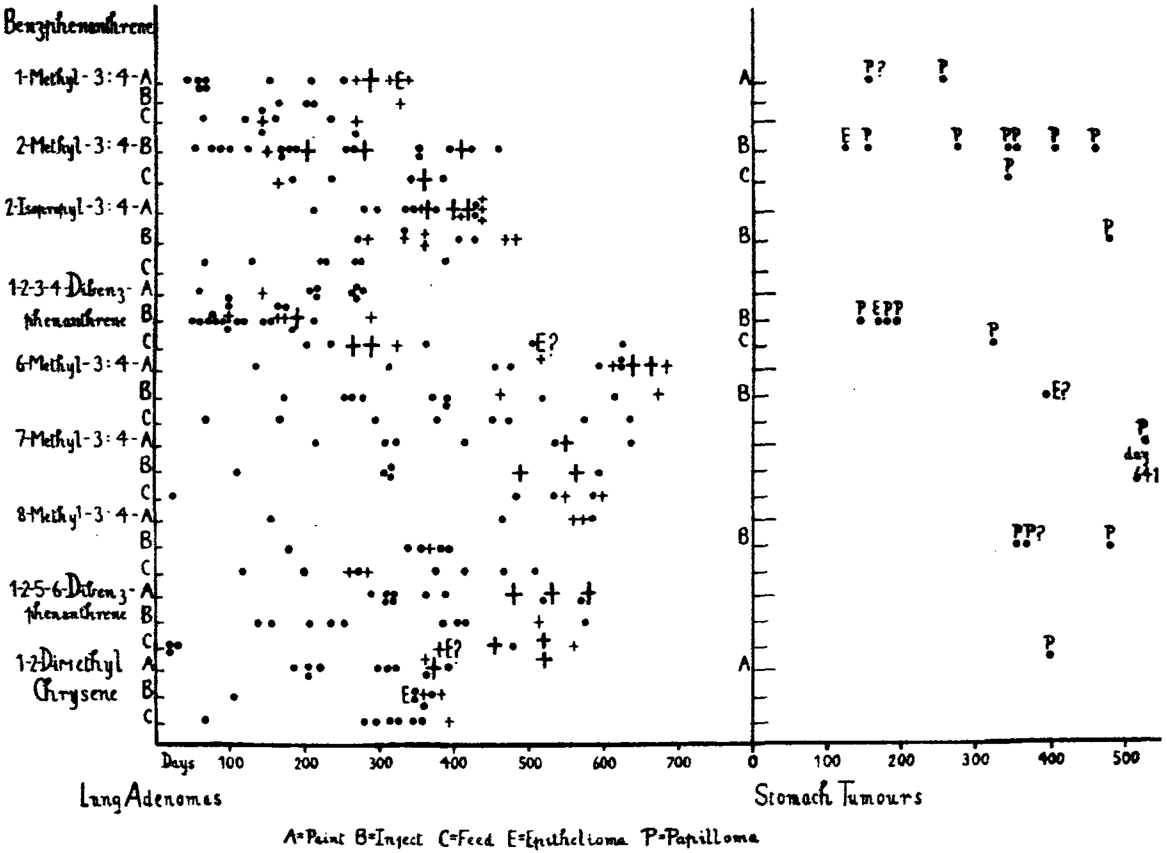

FIGURE 1. Compounds related to 3:4-benzphenanthrene. Tumours of lung and stomach.

TUMOURS OF THE STOMACH

Here only papillomas and epitheliomas of the squamous-celled portion of the stomach are considered; the numbers are of course too small to do more than provide suggestions for further work. The percentage of stomachs examined which showed these squamous-celled tumours is stated in table 9 (see also figures 1 and 2).

TABLE 9. TUMOURS OF THE STOMACH

		I Number of mice examined 100th day and onward	Squamous tumours of stomach				VI % of mice examined (column V as percentage of column I)
			II ? Papilloma	III Papilloma	IV Epithelioma	V Total, Papilloma + epithelioma	
1:2-Benzanthracenes	A, B	384	5	9	1	10	2·6
3:4-Benzphenanthrenes:							
1-, 2-,							
1:2:3:4-Dibenz-	A, B, C	105	1	13	2	15	14·3
6-, 7-, 8-,							
1:2:5:6-Dibenz-	A, B, C	95	1	3	1 ?	3	3·2
1:2:5:6-Dibenzacridine	C	12	0	4	1 + pap.	5	42
Fluorenes	A, B	43	1	0	0	1	2·3
Azonaphthalenes	A, B, C	204	2	1	1	2	1·0

The substituted 1:2-benzanthracenes and fluorenes, and the 6-, 7-, 8-substituted 3:4-benzphenanthrenes, are associated with a low yield (2·3–3·2%) of tumours, which may perhaps be the spontaneous incidence in the stock mice used. A much higher proportion (14·3%) accompanies the administration of the 1- and 2-substituted 3:4-benzphenanthrenes; hence the difference between the two groups of 3:4-benzphenanthrenes is here in the same direction as that found in the case of adenomas of the lung, and proliferation of the bile ducts.

The data are insufficient in number to show which method of administration is the most active in producing tumours of the stomach, but the results given by the 1- and 2-substituted 3:4-benzphenanthrenes (figure 1) do not suggest that introduction by the mouth is always especially effectual. The series in which 1:2:5:6-dibenzacridine was given by the mouth (figure 2) showed stomach tumours in five out of twelve mice, but no comparison can be made because complete autopsies were not carried out when the earlier series (application to the skin, injection *sub cutem*, table 4) were under observation.

DISCUSSION

(1) Many of the results brought forward here are to be regarded as preliminary; it has been impossible to test upon a large number of mice each one of such a large number of compounds. Much further work, with the use of mice of pure lines, is required to elucidate such questions as the relative carcinoma-producing and sarcoma-producing power of various compounds, the incidence of tumours of the lung and stomach, and the mechanism of production of multiple tumours. In this laboratory we are now making trial of the substitution of C_3H for stock mice in such experiments.

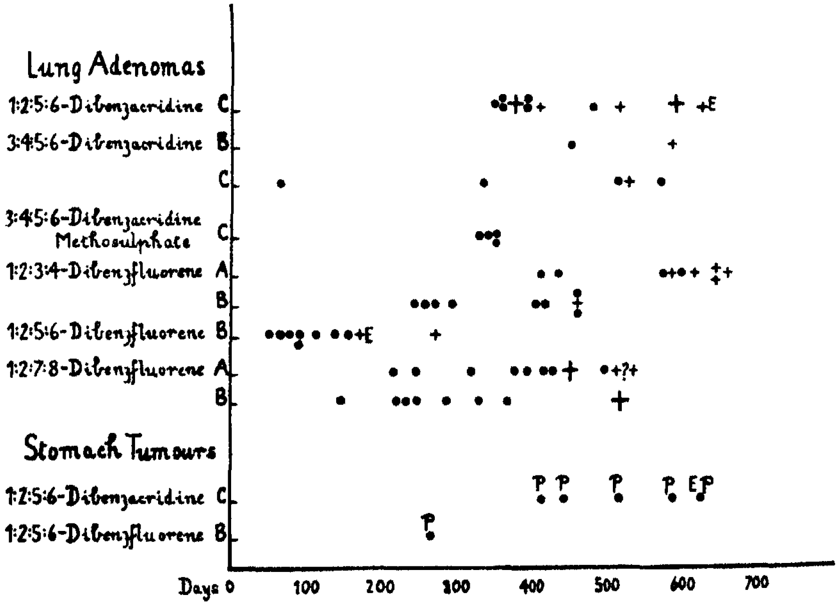

FIGURE 2. Acridine and fluorene derivatives. Tumours of lung and stomach. A = paint; B = inject; C = feed; + = adenoma of lung; ✚ = large adenoma of lung; ● = lung, negative; P = papilloma of stomach; E = epithelioma.

(2) The various histological types of epithelial new growths of the skin of the mouse require further study. All gradations are seen between the purely squamous type and that resembling the rodent ulcer of the human skin. At present we cannot define exactly the part played in the production of these various types by the particular compound, or by the individual animal to which it is applied; the remarks in the last column of tables 1–6 suggest many problems of this kind. In an earlier paper (Bachmann *et al*.

1937) we described and figured mixed tumours of the squamous and rodent ulcer types produced in rats by methylcholanthrene, and similar tumours of the rat produced by 3:4-benzpyrene have been described thus by Oberling, Sannié, M. and P. Guérin (1937): 'L'examen histologique de ces cas a montré des faits très intéressants. Les proliférations cellulaires intéressent presque électivement l'épithélium des follicules pilosébacés et les épithéliomas qui en résultent sont tantôt du type basocellulaire pur, tantôt du type sébacé ou pileux avec ça et là, des ébauches d'évolution malpighienne.' The aetiology of the sebaceous adenoma and its relation to carcinogenic agents requires investigation.

(3) The three modes of administration (by painting, injection *sub cutem*, or feeding) do not give decisive information as to the most effectual method for the production of any particular tumour, because in the two latter some contamination of the fur always occurs; hence inhalation and swallowing cannot be excluded by any of these methods. This consideration affects especially the problem of the experimental production of widespread multiple tumours.

(4) In the present paper the data on the tumours of the lung and stomach and other organs can only be summarized for each compound given; the association together of such tumours in individual mice requires further study.

(5) The 3:4-benzphenanthrene compounds show carcinogenic properties which should be of value in cancer research. They appear to be more active than are the benzanthracenes in the production of multiple tumours, cholangioma, and tumours of the lung, and the 1- and 2-substituted compounds are more active in regard to tumours of the stomach. There is a considerable difference between the compounds substituted on the 1-, 2-side, and on the 6-, 7-, 8-side of the molecule, the former being in most respects more carcinogenic. Among the 1:2-benzanthracenes, the 5-*n*-butyl-, 5-*n*-amyl-, 5-*n*-hexyl group stand somewhat apart from the others, and resemble the 3:4-benzphenanthrenes, in being associated with a higher incidence of lung adenomas, and multiple tumours, and a deficiency in sarcoma production. All the differences suggested in tables 7–9 require testing in mice of pure lines.

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W. E. Bachmann (5-phenyl-, 9:10-diethyl- and 5:9:10-trimethyl-1:2-benzanthracenes, 1:2:benzanthranyl-5-acetic acid, and 3:4-*cyclo*pentenophenanthrene); Dr Ernst Bergmann (1:2:3:4-dibenzfluorene); Professor M. Gomberg (tetraphenylmethane and pentaphenylethane); Dr W. O. Kermack (the three dihydrodibenzacridines, table 4).

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The estimation of small quantities of carbon dioxide in air by the absorption of infra-red radiation

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This paper describes an investigation of the possibility of measuring small amounts of carbon dioxide in air by the absorption of infra-red radiation. It is shown that the method is simple, trustworthy and accurate, and is very sensitive for small amounts of carbon dioxide, of the order of that present in ordinary air. The principle is applied in a form superior to any hitherto used, in that the whole of the transmitted radiation is measured instead of merely the radiation at the maximum of a particular absorption band. This makes a spectrometer unnecessary, eliminates the disturbing effect of variation of temperature, and allows much less sensitive detecting apparatus to be used. The only preliminary treatment necessary is the removal of water vapour from the air under examination.

Introduction

The accurate estimation of small quantities of CO_2 in air streams is a matter of great importance in plant and animal physiology. The usual procedure has been to absorb the CO_2 by an alkaline solution and measure its amount by titration. This method, however, has serious disadvantages when applied to the estimation of very small quantities of CO_2 with which the biologist is often concerned. A considerable improvement was effected when the electrical conductivity of the solution was substituted for the chemical methods of assessing the amount of CO_2 absorbed, thus making more frequent and more accurate estimations possible. This method, first used by Spoehr and McGee (1923), has since been used for micro-estimations of CO_2 by Newton (1935) and Heath (1939), and a modified form of their methods has been used by Nutman (1937) to measure the course of photosynthesis in the field.

An entirely new method was recently introduced by McAlister (1937), depending on the fact that CO_2 has a strong absorption band in the near infra-red, with a maximum at about $4{\cdot}3\,\mu$. By passing a beam of radiation through the air under examination and afterwards through a spectroscope adjusted to measure the transmission at this wave-length, he was able conveniently to measure very small variations of CO_2-content. It was suggested to us by Professor F. G. Gregory, F.R.S., of the Research

Institute of Plant Physiology, Imperial College, that it was very desirable that the possibilities of this method should be systematically explored, and Dr F. J. Nutman, Physiologist to the Amani Research Institute, Tanganyika, then on a short visit to this country, kindly undertook the preliminary work under the direction of one of us and in close consultation with Professor Gregory. Dr Nutman's researches, consisting of investigations into the most practicable source of radiation and form of containing vessel, have been of great value, and the source and tubes used by us throughout were those recommended by him.

PRINCIPLE OF THE METHOD

The absorption band in question is a strong, wide one, and it at once occurred to us that unnecessary demands on the sensitivity of the apparatus were made by restricting the observations to the position of its maximum. The advantage of so doing is that the proportion of radiation absorbed is greatest at this position, but the total amount transmitted is so small that very delicate instruments are necessary to measure it. It happens, however, that the only other constituent of normal air which absorbs the near infrared is water vapour. It is therefore sufficient to dry the air (or, alternatively, insert a water vapour filter in the path of the radiation, but preliminary experiments showed that this was less practicable) in order to make the whole range of radiation available for measurement. It is then necessary merely to measure the total transmission of the air in order to estimate the amount of CO_2 present.

The advantages of this modification are threefold, and are very great. First, as has already been remarked, the detecting apparatus need not be nearly so sensitive. Second, the spectroscope can be dispensed with, thus causing a great simplification and saving of cost. And, third, variation of temperature has a much smaller effect on the total absorption than on the absorption at a particular wave-length, since its chief effect is to shift the position of maximum absorption: the results are therefore subject to far less uncertainty.

We accordingly undertook to investigate the possibility of applying the total infra-red absorption to the estimation of the CO_2-content of ordinary air. The experiments here described show that the method is simple, sensitive, and trustworthy, and the arrangement we have used may be applicable with little modification to some outstanding biological problems. In view of the variety of these problems, however, we have not attempted to work out the most convenient form in which the principle can be applied to any one of them, but have merely sought to establish its validity, to determine its sensitivity, and to give some indication of the degree of delicacy required in the apparatus used.

APPARATUS

The apparatus is shown diagrammatically in figure 1. Two similar stainless-steel tubes, *A* and *B*, each about 180 cm. long and 2·5 cm. internal diameter, were placed in alignment, with a large fish-tail Meker burner (dimensions of grid about 10 cm. × 2 cm.) placed symmetrically between them. The ends of the tubes were closed by dark mica windows held in position by screwed-on caps; a coating of shellac varnish served to make the joints air-tight. Side tubes near the windows enabled the pressure to be varied and CO_2 to be introduced when necessary; a water pump was used for removing air from the tubes. Two Moll thermopiles, T_1 and T_2, were placed, one at the farther end of each tube, and were connected through a galvanometer, *G*, and a variable resistance, *R*, so that the e.m.f.'s opposed one another when radiation was received by the piles. A thick wrapping of cotton-wool round each thermopile and the adjacent window protected the instrument from draughts and stray radiation. The experiments consisted in principle of varying by known amounts the CO_2-content of the air in one tube while keeping that in the other unchanged, and observing the changes of galvanometer deflection. A resistance, *X*, was inserted as shown in the diagram in order that the deflection should be zero when the tubes were similarly filled.

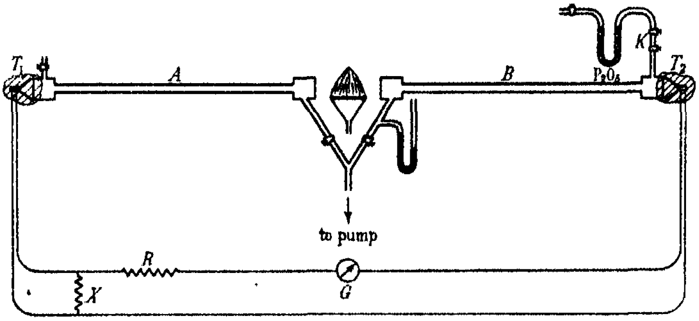

FIGURE 1

Details of the various parts of the apparatus are as follows.

It was found by experience that reflection from the walls of the tubes contributed largely to the quantity of radiation falling on the thermopiles, and stainless steel was found to be a satisfactory reflector. The tubes were therefore made of this material.

Discharges through CO_2 in vacuum tubes were first tried as sources of radiation, but the Meker burner flame proved to be the most satisfactory source. The intensity of the effective radiation was found to be considerably increased when the coal gas was diluted with CO_2—an effect which might be important when only very insensitive detecting instruments are available, but which we found it unnecessary to use. Fluctuations of the flame had little influence on the steadiness of the galvanometer readings, since they affected equally the radiation through both tubes. They were, however, reduced to a minimum by a regulator of the form shown in figure 2, which is self-explanatory, and gave no trouble at all. The regulator served the further purpose of keeping a constant pressure of gas over long periods.

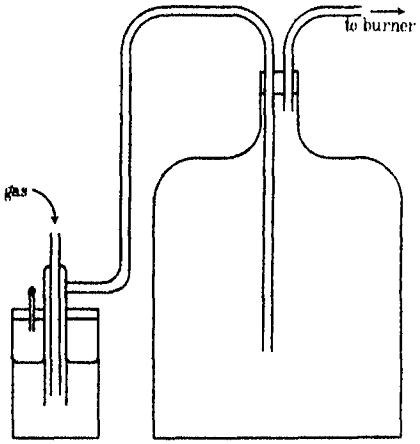

FIGURE 2

Dark mica was used for the windows in preference to quartz because in addition to its relative cheapness it is largely opaque to visible light. The transparency of this substance is practically confined to the region 1μ–5μ, and reaches a maximum near $3{\cdot}7\mu$ (Martin 1919), so that a large proportion of the radiation it transmits is absorbed by CO_2. Messrs F. Wiggins and Sons, of 102–4 Minories, E.C. 3, very kindly supplied samples of East African Biotite, Guatemala Muscovite, and Canadian Biotite for testing. All these substances proved satisfactory, the Guatemala Muscovite being the best, and this material was therefore used in the experiments. The thickness was such that the windows tended to buckle when the pressures on the two sides differed by more than about 15 cm. of mercury, but it was not necessary to exceed this amount in any of the experiments.

The resistance and sensitivity of each of the thermopiles were measured, with the following results:

T_1. Resistance = 34·60 Ω.
Sensitivity to radiation = $3{\cdot}4 \times 10^{-8}$ V per erg per sec.
T_2. Resistance = 31·24 Ω.
Sensitivity to radiation = $3{\cdot}0 \times 10^{-8}$ V per erg per sec.

The values for sensitivity are only approximate, since it was unnecessary to take the elaborate precautions required to obtain precise results.

The galvanometer was a moving coil instrument, having a resistance of 36·3 Ω and giving a deflection of 25 mm. per μA on a scale 95 cm. away.

The general sensitivity of the detecting apparatus is best indicated by the fact that, with a screen in front of tube B and CO_2-free dry air in A, the radiation falling on T_1 gave a deflection of about 360 mm. with resistance $R = 0$ (figure 1).

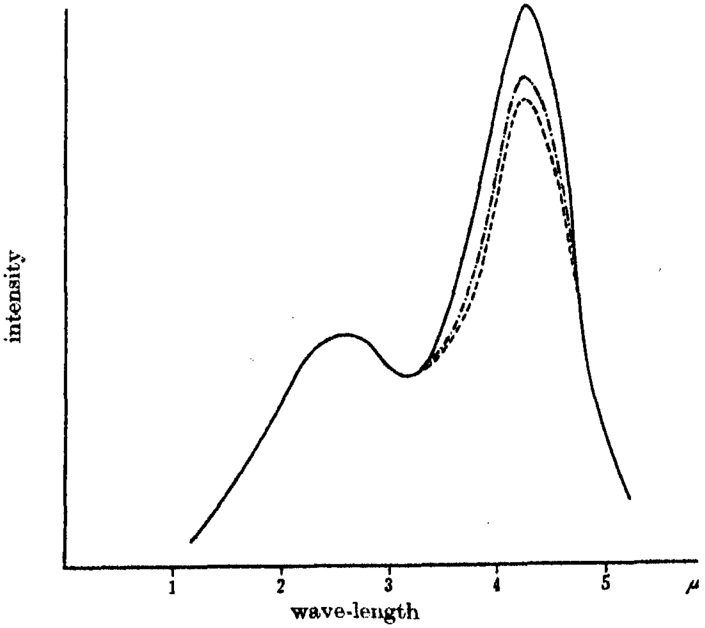

Figure 3

The curves in figure 3 (obtained with a Hilger infra-red spectrometer) show the transmission of radiation of wave-lengths between 1μ and 5μ through one of the tubes when filled with dry air containing 0·0, 0·11 and 0·21 % of CO_2, respectively. The extent and intensity of the CO_2 band are clearly shown.

COURSE OF THE EXPERIMENTS

The procedure adopted was as follows. The galvanometer was first balanced, with both tubes (*A* and *B*) filled with air from which water vapour and CO_2 had been removed by passage through P_2O_5 and soda lime. A quantity of air was then withdrawn from *B* by means of the water-pump (which was connected to the apparatus through a tube containing P_2O_5), the amount being measured by the fall of pressure indicated by a mercury manometer. The pressure was restored to equality with that in *A* by attaching to the inlet tube of *B* a small length of tubing, *K*, between two taps, which had previously been filled with pure dry CO_2 (generated by the action of HCl on potassium carbonate and dried by phosphorus pentoxide), and driving the CO_2 into tube *B* with a current of dried laboratory air freed from CO_2 by passage over soda lime. (The volume of air withdrawn from *B* was, of course, in every case much greater than the volume of *K*.) This method of introduction helped to distribute the CO_2 fairly uniformly throughout the tube. The resulting deflection of the galvanometer was noted. (Alternatively, the change of *X* necessary to restore zero deflection could have been observed.)

This procedure was repeated with various values of the volume, *v*, of *K* and of the pressure reduction, *dp*, in *B*. The deflection was, as expected, found to be independent of *dp* except for slight irregular fluctuations, and to attain a constant value very quickly. A mean deflection was therefore taken for each value of *v*. The results are plotted in figure 4, in which *v* is expressed as a percentage of CO_2 in air at normal pressure.

As a check, a few experiments were made in which the air driving the CO_2 into *B* had not itself been previously freed from CO_2. In these cases, of course, the deflection was not independent of *dp*, since CO_2 was introduced with the laboratory air as well as from *K*. Extrapolation gave the deflection for a pressure reduction corresponding to the removal from *B* of a volume of air equal to *v*, and this was taken as the deflection produced by the CO_2 in *K*. Points in figure 4 so obtained are indicated by crosses, and are seen to give the same graph as the others.

The volume *v* was determined by weighing the mercury necessary to fill *K*, and the various values were obtained by using different tubes and inserting in them pieces of copper wire of known volume.

DISCUSSION OF RESULTS

It will be seen from figure 4 that the method is accurate and trustworthy, and that its sensitivity increases as the CO_2-content of the air diminishes.

Our investigations do not extend to higher concentrations than about 0·2 %, but the curve suggests that fair precision might be obtained over a small range above this value.

No allowance was made for changes of room temperature, which varied between 20·6 and 23·7°C during the experiments. Such fluctuations had no appreciable effect on the readings, so that the method may be considered satisfactory without corrections so far as ordinary temperature changes are concerned. As has been said, this gives it a great advantage over methods depending on variations of absorption at the maximum of the band.

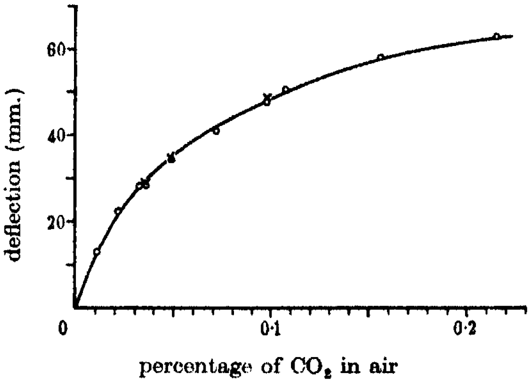

FIGURE 4

It is important, however, to allow the burner to remain alight in position for some time (45 min. was sufficient in our experiments) before readings are begun. This is necessary, apparently, in order that the tubes and mica windows shall attain a constant temperature and the air in the neighbourhood of the flame a constant CO_2-content. Steady conditions having been established, however, there is no difficulty in obtaining consistent results.

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The growth of nodule bacteria in the expressed juices from legume roots bearing effective and ineffective nodules

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Strains of pea and soy-bean nodule bacteria, differing in their effectiveness in benefiting the host legume, were grown in media containing the unheated root juices from uninoculated host plants and from host plants bearing effective and 'ineffective' nodules, and their growth was measured.

The growth of the different bacterial strains on root juice from uninoculated plants was not correlated with their effectiveness.

The juice from roots with effective nodules produced significantly better growth of the bacteria than juice from roots with ineffective nodules in twenty-seven comparisons out of forty-four, the differences in the remaining comparisons being insignificant.

The juice from roots with effective nodules produced significantly better growth than the juice from uninoculated roots in ten comparisons out of twenty-five, and significantly poorer growth in three comparisons.

The juice from roots with ineffective nodules produced significantly poorer growth than the juice from uninoculated plants in eleven comparisons out of twenty-five, and better growth in only one comparison.

The production, as a result of infection, of soluble substances affecting growth of the bacteria, affords an explanation of those differences in nodule growth that determine the effectiveness or ineffectiveness of the different strains of bacteria as regards nitrogen fixation within the host.

Introduction

Different strains of bacteria-producing nodules on a given host legume differ greatly in their ability to benefit that host by fixing nitrogen. Some strains are so 'ineffective' that they produce no appreciable improvement in growth of the legume and fix nitrogen in amounts difficult to detect.

The ineffectiveness of such strains was at first attributed to some defect in the process of nitrogen fixation.

The recent study of clover, pea and soy-bean nodules made by Thornton and Chen (1940), however, has shown that nodules produced by an ineffective strain contain a much smaller volume of infected cells filled with nodule bacteria than do effective nodules, and also that in them, the central tissue containing these infected cells disintegrates after a relatively very short active life. Estimates were made of the mean volume and duration of this central 'bacterial tissue' in nodules of effective and ineffective types. Plants of both clover and soy beans showed large differences in nitrogen content according to which type of nodule they carried, but the quantities of nitrogen fixed by a unit mass of infected nodule cells in unit time was found to be the same for effective and ineffective nodules. The whole of the large differences in total nitrogen fixed could be accounted for by differences in the total volume of infected nodule cells and the duration of these cells prior to disintegration. The further elucidation of the cause of ineffectiveness thus requires that the relatively small total volume and the short life of the infected cells in ineffective nodules be explained.

Nodules produced by effective and ineffective strains develop during their early stages in the same manner, and begin to grow at the same rate. But after a very short while (with clover in about 7 days), the ineffective type of nodule stops growing, while nodules produced by an effective strain continue their growth. This arrested growth is the principal cause of the small volume of infected cells contained in an ineffective nodule, although in soy beans the smaller proportion of infected to sterile cells in the central tissue is a contributory factor. Arrested growth is due to the stopping of cell division in the apical cap of nodule cells. Presumably the initiation and maintenance of the apical meristem is due to growth substances produced by the bacteria. The production of growth substance by the nodule bacteria has been demonstrated by Link (1937) and Thimann (1936, 1939), but Chen (1938) found that effective and ineffective strains of clover bacteria when growing equally vigorously in liquid culture produced similar amounts of growth substance, as assayed by Went's split pea stem test. The stopping of growth in an ineffective nodule would thus seem to be a consequence of the early arrest or decrease in growth of the contained bacteria. Weaker growth of the invading bacteria in ineffective nodules is also suggested by the observation that in soy-bean nodules the central tissue contains a smaller proportion of infected cells in ineffective than in effective nodules.

There is considerable variation in the growth rates of various strains of nodule bacteria in laboratory media, but no correlation could be found between this character and effectiveness towards the host plant. So that the striking difference between the growth of effective and ineffective strains of bacteria within the nodule is not due to any inherent difference in their powers of multiplication, but must indicate that the latter type of strain finds the host tissues an unsuitable environment for its growth.

Disintegration of the central bacterial tissue with its contained bacteria seems eventually to take place in all nodules by whatever strain they are produced, although in effective nodules it does not normally occur until the nodule is getting old. It can, however, be induced in quite young nodules, even of effective strains, by imposing abnormal conditions on the host plants, as by growing these in a boron-deficient medium (Brenchley and Thornton 1925), in the dark (Thornton 1929), or with an excessive nitrate supply (Thornton and Rudorf 1936). In nodules produced by ineffective strains on normally-grown host plants, the disintegration of the central tissue and bacteria also takes place in quite young nodules, and is closely similar to that which can be produced by artificially changing the physiology of the host plant. Like the stopping of nodule growth, the disintegration indicates a maladaptation between host plant and nodule organism.

This maladaptation of inefficient strains to their host plant may be due to any of the following three causes: (*a*) Some factor may be normally present, in the host root system, that is specifically less favourable to the ineffective than to the effective strains. (*b*) The presence of an effective strain within the tissues may induce a change favourable to the growth of the bacteria, which is not induced by the presence of the ineffective strain. Here the action of the induced factor need not specifically favour either type of strain. (*c*) The presence of invading bacteria of an ineffective strain may induce some change in the host tissue that is detrimental to growth of the bacteria. Here again the induced factor need not act specifically against ineffective strains, but these strains must be the specific cause of its appearance.

The substances in solution within the nodule tissue must supply food material to the bacteria so that it seemed natural to search in the root and nodule juice for the factors affecting their growth. On our first hypothesis, (*a*), the root juice of the host plant, whether uninoculated or bearing either type of nodule should be specifically less favourable to the growth of ineffective than to effective strains of the bacteria. On the second hypothesis, (*b*), juice from roots bearing effective nodules should be more favourable to the growth of the bacteria than that from roots bearing ineffective nodules or from uninoculated roots. On the third hypothesis, (*c*), juice from roots bearing nodules containing an ineffective strain should be less favourable to the growth than juice from roots bearing effective nodules or from uninoculated roots.

To test these hypotheses, plants were grown in sterilized sand without inoculation, and inoculated with effective and ineffective strains; the root juices were extracted from each set, sterilized by filtration and their effects on the growth of nodule bacteria of either type were tested *in vitro*. Clover was found to be unsuitable for this work owing to its small root development in the young state. Peas and soy beans were used because these provide ample root material, and since strains showing wide differences in effectiveness have been isolated from their nodules (Helz, Baldwin and Fred 1927; Wright 1925). The cultures of pea and soy-bean nodule bacteria used in this work were supplied by the Wisconsin Agricultural Experiment Station, to whom the authors are gratefully indebted.

TECHNIQUE

The plants were grown at Rothamsted in sand in glazed earthenware pots, the peas in small pots holding 3 kg., and the soy beans in large pots holding 12 kg. of sand. In the experiments with peas, the sand was rendered free from live nodule bacteria by blowing superheated steam through the pots of sand via the aperture at the base. The sand used for experiments with soy beans was not sterilized, since it was found not to contain bacteria capable of producing nodules on soy beans. *In all the experiments described below, whether with peas or soy beans, the uninoculated sets remained free from nodules.*

In the experiments carried out in 1936 and 1937 the following food solution was added to the sand, to about 15 % of its dry weight, and fresh supplies were given during the growth of the plant:

K_2SO_4	0·87 g.	$CaSO_4$	0·2 g.
K_2HPO_4	0·3 g.	$FeCl_3$	0·04 g.
KH_2PO_4	0·3 g.	$MnSO_4$	0·04 g.
NaCl	0·5 g.	Boric acid	0·04 g.
$MgSO_4.7H_2O$	0·5 g.	Tap water	1 litre

For the 1939 experiments this food solution was modified as shown below, so as to give a better calcium supply.

K_2SO_4	0·9 g.	$FeCl_3$	0·04 g.
K_2HPO_4	0·5 g.	$MnSO_4$	0·04 g.
$CaH_4(PO_4)_2.4H_2O$	0·5 g.	Boric acid	0·04 g.
$MgSO_4.7H_2O$	0·5 g.	Tap water	1 litre
NaCl	0·5 g.		

The food solutions were sterilized before addition to the pots, and in the experiments with peas all watering was with boiled water.

In all experiments, inoculation was accomplished by mixing a suspension of the appropriate organism with the food solution before this was poured into the sand, the uninoculated pots receiving an equal volume of sterile water in place of the bacterial suspension. The seeds were externally sterilized by immersion in absolute alcohol followed by 0·2 % aqueous $HgCl_2$ for 3 min., and then washed in four changes of sterile water. They were sown immediately after application of the food solution to the pots. When the plants reached the flowering stage, the roots were shaken free from sand, washed, and the surplus water drained off them. They were then minced, ground in a mortar and the juice squeezed through muslin. This juice was then passed through two thicknesses of Whatman no. 4 filter-paper, and further cleared by filtration under reduced pressure through a pad 2 in. thick of compressed filter-paper fragments set up in a wide glass cylinder.* To prepare this pad, wet pieces of filter-paper about 1 cm. square were compressed with a glass ramrod. In the 1937 experiment with soy beans this filter was replaced by a Seitz filter.

After clearing, the juice was rendered sterile by filtration successively through an L1 Chamberland filter-candle, and through a sterilized L5 candle set up in a sterile vacuum flask.

The effect of the various root juices upon the growth of the nodule bacteria *in vitro* was tested by two different methods. In the 1936 experiments with soy beans and peas the juices were added to a melted agar medium which was poured into petri dishes and allowed to set. A number of point inoculations of the test organism were made on each plate, and the mean area of the resulting colonies was taken as the measure of growth.

In later experiments the test organisms were grown in liquid media containing the root juices, and their growth was measured by haemocytometer counts. As the details of technique varied somewhat in the different experiments they will be described with each experiment.

EXPERIMENT WITH SOY BEANS, 1936

Five sets of soy beans were grown in pots of sand. One set was uninoculated and the remaining sets inoculated with nodule bacteria of the effective strains 501 and 505, and of the ineffective strains 502 and 507. Six parallel pots, each with eight plants, were set up and growth carried out from 1 September till 26 October.

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* The apparatus used is illustrated in *Proc. Roy. Soc.* B (1936), **119**, figure 1, p. 478.

The roots were washed and the juice extracted and sterilized by filtration. The medium used for testing the effects of the juices on bacterial growth had the following composition:

K_2HPO_4	1·0 g.	$FeCl_3$	0·02 g.
KH_2PO_4	1·0 g.	Sucrose	10·0 g.
$MgSO_4.7H_2O$	0·4 g.	Yeast water*	100 ml.
NaCl	0·2 g.	Tap water	900 ml.
$CaCO_3$	1·0 g.	Agar	20·0 g.

This basal medium was divided into five portions which were autoclaved, cooled to 42° C and, to each portion, one of the juices was added with a sterile pipette to make 18 % of the final volume. From each of the four media containing juice from the inoculated plants, sixteen petri dish plates were poured, each of 10 ml. of medium. These were allowed to set and incubated at 25° C for 24 hr. to allow the agar surface to dry. Each batch of sixteen plates were then divided into four sets of four replicate plates, one set being inoculated with each of the four strains of soy-bean nodule organisms 501, 505, 502 and 507.

In the case of the uninoculated roots, however, the small supply of juice enabled only three replicate platings to be inoculated with each test organism.

By the above plan each of the four strains was grown on media containing each of the five juices, namely, from uninoculated plants and from plants that had borne nodules produced by each of the same four strains (see table 1).

The inoculation of the agar plates was carried out as follows. Ten needles were set about 1·5 cm. apart in a large cork, with their points projecting and carefully adjusted so that the points were level. The culture to be used as inoculum was suspended in sterile saline solution, and about 10 ml. were poured into a sterile petri dish. The needles, sterilized by flaming, were lowered into the suspension, and their points then gently brought down on to the surface of the agar medium in the plate. In this way each plate was given ten point inoculations. After incubation for 14 days at 25° C, the colony areas were measured. The results are shown in table 1, which gives the mean colony areas in square millimetres. The mean colony area on each plate was taken as the unit in calculating the standard error. The number of degrees of freedom for each set is given in the table. This is not always three, because less than four replicate plates with juice from uninoculated plants were tested with each strain, and because some plates in other sets were lost from various causes. In particular, the set in which strain 502 was tested on juice 501 was spoilt owing to water condensing on the agar surface. In order to obtain a valid standard error for the whole experiment, a hypothetical value was allotted to this set, calculated by Yates's method for missing plots (Yates 1933). The standard error for the differences between any two individual set means is ± 1·38. The bottom row gives the mean colony areas for the four test organisms combined in the case of each juice tested. The standard error for the differences between any two of these juice means is ± 0·69.

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* Yeast water was made by boiling 10 % yeast in water and filtering.

TABLE 1. EXPERIMENT WITH SOY BEANS, 1936

Colony areas of four strains of soy-bean nodule bacteria on agar media containing root juice from variously treated plants

Strain	Mean colony area sq. mm. (*m*) Degrees of freedom (*n*)	Medium containing root juices from				
		Uninoculated plants (control juice)	Plants inoculated with strain			
			501	505	502	507
501	*m*	4·71	6·75	11·35	0	0
	n	1	3	3	3	3
505	*m*	8·58	6·06	14·04	6·27	8·17
	n	2	3	3	2	2
502	*m*	8·49	(11·52)	19·56	6·22	10·96
	n	1	0	4	3	2
507	*m*	6·28	7·07	7·99	5·18	4·37
	n	0	3	2	3	3
All strains	*m*	7·02	7·85	13·23	4·41	5·87

S.E. of difference between any two individual means = ± 1·38.
S.E. of differences between juice means (bottom row) = ± 0·69.

The relative effect on the bacterial growth of root juice from plants bearing effective nodules, as against root juice from plants with ineffective nodules, can be tested in fourteen comparisons, excluding, of course, the hypothetical value. Juice from plants bearing the effective strain gave significantly the better growth in ten of these comparisons.

The root juice from plants bearing 501 nodules did not differ significantly from the juice of uninoculated roots (described below as 'control juice') in its effects on any of the strains tested. Root juice from plants bearing the other effective strain 505, however, gave significantly better growth than did control juice with three of the test organisms and no significant difference with the fourth.

Juice from roots bearing ineffective nodules of strains 502 and 507, completely prevented growth of strain 501, but with the other three test organisms these two juices produced growth not significantly different from control.

In this experiment the results are suggestive, but the difference in action of the two juices from plants with effective nodules and the different response of the four test organisms to the juices from plants with ineffective nodules, makes the comparison with control juice inconclusive.

EXPERIMENT WITH PEAS, 1936

In this experiment peas, inoculated with the effective strains 310 and 317, with the ineffective strains 313 and B 33 and uninoculated, were grown in small pots of sand as described above. The seeds were sown on 1 September and the plants reaped on 21 October. The root juices were extracted, and the growth of the four strains of pea-nodule bacteria on agar media containing each of the juices was tested by the same method as in the previous experiment. Each organism was, in addition, grown on the basal agar medium in which the root juice was replaced by distilled water. The results are shown in table 2 in which the mean colony areas are derived from three to five replicate plates, each bearing ten colonies. The standard error for the difference between any two individual means is ± 18·38, while that for the difference between the juice means given in the bottom row is ± 9·19.

In this experiment, juice from plants with efficient nodules gave significantly better growth than juice from plants with inefficient nodules in eight out of the sixteen possible comparisons and insignificant results in the remainder.

In comparison with the uninoculated control juice, that from roots bearing one of the effective strains (310), has given variable results, and its mean effect on all four test organisms does not differ significantly from that of the control juice. Roots bearing the other effective strain (317) have produced a juice giving significantly better growth of two test organisms, as compared with control juice, but insignificant results with the other two. Both the root juices from plants bearing ineffective nodules (strains 313, B 33) produced poorer growth than control juice with every test organism, although these differences were significant in only three out of the eight comparisons with individual test organisms. But when the growths of the four test organisms are combined, the growth on these two juices differs from that on control juice with a high degree of significance. In only one comparison out of the eight did these two juices produce significantly better growth than the medium without any root juice at all.

TABLE 2. EXPERIMENT WITH PEAS, 1936

Colony areas of four strains of pea-nodule bacteria on agar media with and without root juices

Strain	Mean colony area sq. mm. (*m*) Degrees of freedom (*n*)	Medium containing root juices from					Medium without root juices
		Uninoculated plants (control juice)	Plants with nodules by strain				
			310	*317*	*313*	B33	
310	*m*	153·4	198·6	190·2	106·3	151·3	102·5
	n	3	3	3	3	4	4
317	*m*	159·4	114·2	145·3	104·3	112·2	94·0
	n	2	3	3	3	2	3
313	*m*	105·6	103·3	156·7	92·5	94·3	90·0
	n	2	3	3	3	3	4
B33	*m*	130·6	124·1	148·1	94·7	113·6	112·6
	n	3	3	3	3	3	3
All strains	*m*	137·3	135·0	162·3	94·4	117·8	99·7

S.E. of differences between individual means = ± 18·38.
S.E. of differences between juice means = ± 9·19.

This experiment thus confirms the conclusions of the first experiment in that plants bearing one of the effective strains (317) produced a juice stimulating bacterial growth compared with control juice, while the juice from plants bearing either of the ineffective strains was definitely less good than control juice as a medium for the growth of the test bacteria.

EXPERIMENT WITH PEAS, 1937

The method of estimating bacterial growth by colony area was found to be not altogether satisfactory owing to uneven growth of the colonies; it was, therefore, decided to estimate growth by means of haemocytometer counts of the test organisms growing in liquid culture.

Peas were grown in small pots of sand, sets of eight replicate pots being inoculated with strains 310, 317 and B33, and one set left uninoculated. The peas were grown from 8 March till 6 June, and the root juices extracted and sterilized by filtration. The test organisms were grown in the following liquid medium:

K_2HPO_4	0·5 g.	$CaCO_3$	0·5 g.
KH_2PO_4	0·5 g.	Sucrose	10·0 g.
$MgSO_4.7H_2O$	0·2 g.	Yeast water (10 % yeast)	100 ml.
NaCl	0·1 g.	Tap water	900 ml.
$CaSO_4$	1·0 g.		

The medium was put up in 20 ml. portions in small flasks, sterilized in the autoclave and 5 ml. of juice added aseptically to each flask. Duplicate flasks of medium containing each type of juice were inoculated with standard loopfuls of a suspension of B33 strain of pea-nodule bacteria. After 18 days' growth at 25° C, the numbers of bacterial cells were counted on a haemocytometer ruled in $\frac{1}{400}$ sq. mm. The results are set out in table 3, where each value given is the mean count of duplicate flasks. The standard error for the difference between any two means is ± 25·9.

TABLE 3. EXPERIMENT WITH PEAS, 1937

Growth of pea organism, strain B33. Bacterial cells per ml. in liquid media containing root juices, means of duplicate cultures

Medium containing root juices from peas			
Uninoculated (control juice)	Inoculated strain 310	Inoculated strain 317	Inoculated strain B33
1828	2083	1915	1608

S.E. of difference between any two means = ± 25·9, $n = 4$

Both the efficient strain root juices gave significantly better growth than did the control juice. The juice from roots bearing the inefficient B33 nodules produced less growth than any of the other juices, the significance of the differences having in each case a probability of less than 1 %.

EXPERIMENT WITH SOY BEANS, 1937

Soy beans were grown in large pots with one set uninoculated and other sets inoculated with strains 501, 502, 505 and 507. The seed was sown on 10 April and the plants reaped on 10 July. Root juices were extracted and sterilized as before. A liquid medium was made up similar to that used in the last experiment but with the sucrose replaced by dextrose, which was found better for the growth of soy-bean bacteria. This medium was put up in 15 ml. portions in wide test-tubes, to each of which, after sterilization, 5 ml. of juice to be tested was added aseptically. Batches of six replicate tubes were given control juice, and juices from plants bearing 505 and 507 nodules respectively. Two of each of these tubes were inoculated with strain 501, two with strain 502 and two with strain 507. The yield of juice from plants bearing 501 and 502 nodules was insufficient, so that only two strains of test organisms, 501 and 502, were grown on these juices, each in duplicate tubes..

After 12 days' incubation at 25° C, the growth of the bacteria was estimated from haemocytometer counts. The results of this experiment are shown in table 4.

TABLE 4. EXPERIMENT WITH SOY BEAN, 1937

Growth of soy-bean organisms. Bacterial cells per ml. in liquid media with root juices, mean of duplicate cultures

Test organism	Medium containing root juice from soy beans				
	Uninoculated (control juice)	Inoculated with strain			
		501	505	502	507
507	1251	—	790	—	668
	S.E. of difference between any two means, 65·1, $n = 3$				
501	1835	1938	1533	403	960
502	1680	2122	1263	1328	660
Means of both strains	1757	2030	1397	866	810

S.E. of the difference between individual means $= \pm 210{\cdot}7$, $n = 10$

On account of the incompleteness of the test with 507 bacteria, separate standard errors have been calculated for this series and for the remaining two series inoculated with 501 and 502 bacteria. With the former, the standard error for a difference between the means is ± 65·1, while for the latter the error for differences between individual means is ± 210·7.

When growth on juices from plants bearing efficient and inefficient strains is compared for the three test organisms, the inefficient strain juices give significantly the poorer growth in seven, and insignificant differences in two of the nine comparisons.

Five comparisons can be made between growth of the test organisms on juices from plants bearing effective nodules of strains 501 and 505 with their growth on juice from uninoculated plants. In one comparison the former type of juice gave significantly better and, in a second, significantly poorer growth. The other differences were not significant. The two root juices from plants bearing inefficient nodules of strains 502 and 507 gave significantly poorer growth than did the control juice in four comparisons out of five, and gave an insignificant result in the remaining comparison.

This experiment thus confirmed the depressing action of juices from roots with inefficient strains, but did not bear out the previous experiments as regards the improved growth on juices from roots with the efficient nodules as compared with control juice.

EXPERIMENT WITH PEAS, 1939

Peas were grown in small pots of sand with one set uninoculated and others inoculated with strains 317 and 313. The plants were grown from 4 April till 26 June, and the root juices extracted and filtered as before. Small flasks were set up, each containing 50 ml. of the following medium in which the yeast extract was replaced by asparagin as a nitrogen source, so that the root juice should supply the sole source of accessory growth substances.

K_2HPO_4	0·5 g.	$CaSO_4$	0·5 g.
KH_2PO_4	0·5 g.	$FeCl_3$	0·02 g.
$MgSO_4.7H_2O$	0·2 g.	Asparagin	0·5 g.
NaCl	0·1 g.	Dextrose	5·0 g.
$CaCl_2$	0·1 g.	Tap water	1000 ml.

The flasks of medium were sterilized, and to each 10 ml. of root juice were added aseptically. Each flask was inoculated with 1 ml. of a suspension of the test organism. Eight flasks of control juice and six of each of the other types of juice were set up, and half the flasks with each juice were inoculated with 317 and half with B33 organisms. Two of the flasks inoculated with strain B33 and one inoculated with strain 317 were lost by contamination, so that the number of replicates counted varies between the different sets as shown in table 5. The results of the haemocytometer counts after 10 days' incubation at 25° C are shown in table 5.

The growth of strain 317 was poor on all media, and did not make significantly different growth on any of them. Organism B33 grew better. Its growth was much poorer on juice from roots bearing inefficient 313 nodules than on control juice, but growth was also significantly poorer on the 317 juice. Indeed, the two juices from inoculated roots did not differ significantly from each other in their effects in this experiment.

Table 5. Experiment with peas, 1939

Growth of pea-nodule bacteria, strains 317 and B 33. Bacterial cells per ml. in media with pea-root juices

Test organisms	Bacterial numbers (m) Degrees of freedom (n)	Media containing root juices from peas: Uninoculated (control juice)	Inoculated with strain 317	Inoculated with strain 313
317	m	169	145	156
	n	3	2	2
S.E. of difference between two means = ± 12·73, $n = 7$				
B 33	m	2079	1516	1295
	n	2	1	2
S.E. of difference between two means = ± 129·8, $n = 5$				

Experiment with soy beans, 1939

Soy beans were grown in large pots of sand with one set uninoculated and other sets inoculated with strains 501 and 507 respectively. The seed was sown on 4 April and after 10 weeks' growth the roots were washed and the root juices extracted and sterilized by filtration. The same liquid test medium was employed as in the last experiment with peas; duplicate flasks were set up with each type of juice, 10 ml. of juice being added aseptically to 50 ml. of sterilized medium. All the flasks were inoculated with 1 ml. of a suspension of strain 507 and incubated at 25° C for 3 weeks, growth of the bacteria being slow. Haemocytometer counts of the resulting growth gave the mean figures shown in table 6.

Table 6. Experiment with soy beans, 1939

Growth of soy-bean nodule bacteria, strain 507. Bacterial cells per ml. in media containing soy-root juices. Means of four replicate cultures

Medium containing root juice from plants: Uninoculated (control juice)	Inoculated with strains 501	Inoculated with strains 507
471	1980	1127

S.E. of difference between means = ± 129·8, $n = 9$

The juice from roots bearing the inefficient 507 nodules gave significantly poorer growth of the test organism than did the juice from roots with the efficient 501 nodules, but the control juice behaved in an unusual manner and gave much poorer growth than either of the other juices. It is possible that this unusual result was due to the omission of yeast from the basal medium. This experiment thus provides the only case in which juice from plants bearing nodules of an inefficient strain gave better growth than juice from nodule-free roots.

DISCUSSION

The composition of root juice is clearly liable to be affected both by growing conditions of the plant and by details in the method of extraction and filtration that are very difficult to control. It is to be expected, therefore, that somewhat variable results will be obtained in experiments of the type described above. The experiments must be considered together in order to assess the validity of conclusions derived from them. They comprise six experiments, three with peas and three with soy beans. The fact that the technique of testing the effect of the juices was varied from one experiment to another will strengthen the conclusions common to all the experiments. In most of the experiments several test organisms were used, and in all of them several types of root juices were compared.

It was pointed out in the introduction (p. 477) that the maladaptation to their host plant shown by ineffective strains might be due to some factor normally present in the root system, that acted specifically against such strains. The growth of the different bacteria in root juice from uninoculated plants, shown in tables 1, 2, 4 and 5, is not correlated with the effectiveness or otherwise of the strain used as test organism. This shows that the root juice does not normally contain any factor that acts specifically against ineffective strains.

On the other hand, there is evidence that the type of nodule on the root system affects the ability of the juice from roots and nodules to support growth of the test organisms. Analyses of variance were made for each experiment, and the standard errors of differences between means obtained. The latter are given for each experiment. From them has been calculated the significance of differences in growth of the nodule bacteria on juices from plants bearing effective and ineffective nodules, and on juice from nodule-free roots. These comparisons have been summarized in table 7, in which a 'plus' sign means that there has been significantly better, and a 'minus' sign significantly poorer growth, while '0' means that the difference did not attain significance. The third column shows the comparative growth of test organisms on juices from roots bearing effective nodules as against growth on juices from roots with ineffective nodules. Out of the forty-four possible comparisons, there were twenty-seven in which the former juice gave significantly the better growth. In all remaining seventeen cases the differences were insignificant.

This effect of root juice upon bacterial growth affords a simple explanation of the observed appearances in effective and ineffective nodules. The rapid growth and long duration of activity amongst the bacteria in effective nodules and the slower growth and early onset of disintegration of the bacteria in ineffective nodules is just the result that might be expected from the different environment provided by the root juice in each case.

The difference in effect of juice from roots bearing effective and ineffective nodules might itself be due to a stimulating action upon bacterial growth of the former type of juice, or to a depressing action of the latter type. These two effects can, to some extent, be separated by comparison with the effects of control juice from nodule-free roots.

Twenty-five comparisons have been made between the effects of juices from roots bearing effective nodules and those of control juice (column 4 of table 7). The former gave significantly better growth of bacteria than control juice in ten cases and significantly poorer growth in three cases.

Twenty-five comparisons were made between growth on juices from plants with ineffective nodules and on control juice (column 5, table 7). In eleven of these the juice from roots with ineffective nodules gave growth significantly poorer than control juice, and in only one case was this result reversed.

The evidence taken as a whole thus indicates that there is an increased stimulating effect from juices of roots bearing effective nodules, possibly connected with the products of nitrogen fixation, while the presence of ineffective strains in the roots induces the appearance of some factor in the root juice which is depressing in its action on bacterial growth.

With regard to the nature of this depressing factor, it should be noted that the test organisms made growth on all the juices that were tested except on two in the first experiment. In no instance could a transmissible lytic agent be found in the juice. This provides strong evidence against the hypothesis that any of the strains tested owe their ineffectiveness to the presence of bacteriophage. Had bacteriophage been present in a concentration sufficient to produce the symptoms observed in the ineffective nodules, the technique of growing the same strain in the presence of filtrates of the crushed nodules and roots should have revealed its presence. The same technique of juice extraction has, in fact, been successfully used to demonstrate the presence of bacteriophage in nodules of peas grown in garden soil.

The present results provide no evidence as to whether the factors affecting the growth of nodules and of their contained bacteria are localized in those nodules or distributed throughout the root system. But it seems probable that they are localized in or near the nodules; for, *when nodules of both effective and ineffective types develop on the same root system, the characteristic size and appearance of each type of nodule is not affected by the presence of the other type on the same root.*

TABLE 7. SUMMARY OF SIGNIFICANT DIFFERENCES

		3	4	5
		Comparative growth of bacteria on root juices from plants inoculated with		
Experiment	Test organism	Effective strain—Ineffective strain	Effective strain—uninoculated	Ineffective strain—uninoculated
Soy 1936 (table 1)	501	+ + + +	+ 0	− −
	505	+ + 0 0	+ 0	0 0
	502	+ +	+	0 0
	507	+ + 0 0	0 0	0 0
Pea 1936 (table 2)	310	+ + + +	+ +	− 0
	317	+ 0 0 0	− 0	− −
	313	+ + 0 0	+ 0	0 0
	B33	0 + 0 0	0 0	0 0
Pea 1937 (table 3)	B33	+ +	+ +	−
Soy 1937 (table 4)	501	+ + + +	0 0	− −
	502	+ + + 0	+ 0	− 0
	507	0	−	−
Pea 1939 (table 5)	317	0	0	0
	B33	0	−	−
Soy 1939 (table 6)	507	0	+	+
Totals:				
Significantly greater		27	10	1
Significantly less		0	3	11
Insignificant		17	12	13
Total comparisons		44	25	25

A plus sign means that the first-named root juice produced significantly better, and a minus sign significantly poorer growth than the second-named juice. '0' indicates an insignificant difference.

The evidence as a whole suggests that a soluble substance is formed within the nodules produced by an ineffective strain, that is harmful to the growth of the bacteria. The formation of such a substance would account for the early arrest in nodule growth and for the rapid onset of disintegration of the contained bacteria.

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Mutation effects of ultra-violet light in *Drosophila*

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Methods are described whereby the frequency of mutations induced in *Drosophila* spermatozoa by ultra-violet light may be brought to approximately 5 %. Among the means used to increase the mutation rate was the selection of those individuals in which, as shown by their earlier death, the radiation had penetrated more effectively.

It has been shown by the use of a combination of filters that the region in which the mutation-inducing effect of ultra-violet light begins lies between 320 and 300 mμ, as expected for nucleic acid absorption.

Further results, with an improved genetic technique whereby the production of gene mutations and gross rearrangements could be studied in the same individual, have confirmed our earlier conclusion that, as compared with X-rays of the same gene-mutational strength, ultra-violet rays are ineffective in causing gross structural changes of chromosomes.

Preliminary evidence was obtained that ultra-violet rays are similarly ineffective in the production of minute structural changes, and that (in *Drosophila*) they also fail to produce, by 'simple breakage', chromosome fragments that are capable of surviving ('terminal deficiencies').

It may be concluded that activation of the nucleic acid by ultra-violet is ineffective in producing breakage of the chromosome, although it is effective in producing gene mutations.

The gene mutations produced by ultra-violet must occur secondarily, as a consequence of transfer of energy from the nucleic acid, resulting in turn in a change of the distinctive gene material, which is probably of protein nature. It is therefore illegitimate to calculate the size or number of genes from the frequency with which gene mutations are produced by a given dose of radiation.

The gene-mutational change apparently differs in some essential way from the change involved in demonstrable minute rearrangements, but the minute rearrangements appear to be essentially similar to the gross rearrangements, involving a similar kind of thoroughgoing breakage of the chromonema.

The effect of ultra-violet in discriminating between gene mutations on the one hand and both minute and gross rearrangements on the other hand, lends some support to the conception that the 'genes' represent definite segments of the chromonema, and that the connexions between the parts of these segments are different in kind from the connexions between the segments themselves.

A possible means of attack on the mutation theory of cancer, derived from the above conclusions regarding the mechanism of action of ultra-violet light, is pointed out.

1. INTRODUCTORY

(a) *The problem*

The important finding by Altenburg (1930 et seq.) that ultra-violet light produces mutations soon took on additional interest, when he obtained results (1931, 1933, 1936) suggesting that the action of this radiation, unlike that of X-rays and radium, might be discriminatory, in producing gene mutations without gross structural changes in the chromosomes. These results, obtained with *Drosophila*, were as yet inconclusive on the latter point, although there could be no doubt that gene mutations were produced by ultra-violet light. More recently, however, Stadler and his co-workers (1936, 1938) have been able to prove the discriminatory nature of the ultra-violet effect in maize pollen, and since then the present authors (1939) have presented proof of it in *Drosophila*, using that type of cell—spermatozoa—which in the case of X-ray treatment is most susceptible to the production of gross structural changes.

In the present experiments, these results on *Drosophila* spermatozoa have been extended (*a*) by increasing the mutation rate by certain devices; (*b*) by further restricting the range of wave-lengths used; (*c*) by so modifying the genetic technique as to allow both the tests for gene mutation and for gross changes of the translocation type to be carried out on offspring of the same irradiated individuals, as well as by conducting some tests for a type of gross structural change—deletion—which had not been looked for in our previous work; (*d*) by attempting to detect minute structural changes, which arise in a somewhat different manner from the gross ones when X-rays are applied (Belgovsky 1939; Muller 1938, 1940). The latter question is of special importance at present in relation to the problem recently raised (see Muller 1937, 1939; Goldschmidt 1937, 1938) as to whether there is really any essential difference between minute structural changes and 'gene mutations', and whether the individual gene can be regarded as a definitely delimited body.

(*b*) *The ultra-violet source and the measurement of dosage*

The ultra-violet lamp was, as before, a water-cooled capillary mercury arc (Daniels, Farrington and Heidt 1932) mounted in a metal case provided with a window 5 cm. square of Schott and Genossen U.G. 2 glass, 1 mm. thick, which served to filter out all waves shorter than 280 mμ and most of the visible and infra-red. An accessory filter of silver deposited on quartz was sometimes used to limit the ultra-violet spectrum further (see § 2 (*b*)). The energy was measured by a thermopile—or in some cases by a photo-electric cell, calibrated against the thermopile—placed at a standard distance, usually 40 cm., from the source.* A large aperture, high-transmission spectrograph with a simple quartz train and ethyl cinnamate prism was used for determining certain spectral intensities (e.g. table 4).

(*c*) *Method of treatment*

The young adult male *Drosophila* used should be not more than 24 hr. old and of stock with lightly pigmented body and testis. They are anaesthetized and, following the method of Reuss (1935), laid ventral side up on a layer of cotton-wool about 1 cm. thick and 5 cm. square, backed with a similar sized sheet of metal or glass. About 70–100 flies can be placed on this area without undue crowding. A quartz plate 5 cm. square and about 3 mm. thick is laid carefully upon the flies and secured in place with rubber bands, which have sufficient tension to flatten the insects appreciably: the more resistant thorax sinks into the cotton-wool, while the abdominal region is flattened and the gonads brought nearer to the ventral surface. Masking of the head and thorax gives no marked improvement. If the pressure is suitably adjusted the flies can be rendered immobile for several hours without causing appreciable mortality. The flies are placed at a suitable distance from the source (5–15 cm.), so that, using both filters together, a dose of 2×10^{10} ergs/cm.^{2} is administered in about 50–100 min.

The above-mentioned dose of 2×10^{10} ergs/cm.^{2}, where both filters are used, or its equivalent of about 4×10^{11} where only the glass filter is used, as explained in § 2 (*a*), appears to be the optimum for lightly pigmented flies. It is the dose which, with these conditions of treatment, usually gives the best combination of high mutation effect, as detected in the descendants, with not too high mortality of the treated individuals themselves. The desired mutation effect and a practically completely lethal effect on the treated flies are separated by only a narrow margin. If the dose is doubled, practically all the treated flies are sterilized and the great majority die within 24 hr. The frequency of mutants detected in the descendants of the few survivors is then higher (6–9 %), but on account of the small total numbers the actual number of mutations found is very small with the doubled dose. If, on the other hand, the dose is halved, all the flies survive, but mutations are inconveniently rare. At the optimum dose, about 3 % is the mutation frequency for sex-linked lethals which may be expected as a reasonable average under the above conditions of treatment and with the flies used by us, although rates as high as 5 % may occasionally be obtained at this dose.

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* We are indebted to Dr E. B. Ludlam, of the Chemistry Department, for the loan of measuring apparatus and a secondary standard of energy measurement.

Different types of *Drosophila* show distinct variations in effect. Thus, in the case of yellow-bodied flies, a given dose, of approximately optimum size, will produce about the same or a slightly greater mutation effect than, say, a dose 25 % higher when applied to flies of wild-type coloration (see table 3). In the latter, receiving the larger dose, mortality is about 30 % higher; hence a somewhat greater number of flies must be irradiated to obtain a comparable number of survivors. In treating any new variety, it is best to make some preliminary trials: if a certain dose kills some 75 % in 24 hr., three-quarters of this dose will give a reasonable mutation rate with moderate mortality, provided the fly does not have excessive body pigment and has the lightly pigmented testis sheath associated with white eye, with the light alleles of white, and with certain other mutant eye colours.

2. EXPERIMENTS

(a) *Improvement of the mutation rate by selection in vial cultures*

Attention was drawn to this possibility by certain experiments on fractionation of the dose. It is known that with X-rays (Patterson 1931 and others) the mutation rate induced in spermatozoa is independent of fractionation, and dependent solely on the total dose. Many other biological effects of X-rays, however, are reduced by fractionation of the dose. This suggests that perhaps a higher maximum dose of ultra-violet light might be tolerated, and the mutation rate thus raised, if the dose were fractionated. Table 1 shows the result of dividing a dose larger than the standard one into two or three portions, delivered on successive days.

TABLE 1. IRRADIATED *y w* MALES × *ClB* FEMALES

(Approximately 200 flies treated in each case.)

Total dose $\times 10^{10}$ ergs/cm.^{2}*	Arrangement of dose at 24 hr. intervals	Proportion of 24 hr. survivors among treated P_1	Fertile F_1 cultures	Lethals
2·3	1·8 + 0·5	1·0	186	4
2·55	1·8 + 0·75	1·0	234	4
3·0	1 + 1 + 1	0·7	10	0
4·0	2 + 2	0·5	Nil	Nil
4·50	2 + 1·5 + 1	0·3	Nil	Nil
5·8	3·0 + 2·8	0·0	Nil	Nil

* In tables 1, 2, 3 and 6, and in the text, the dose is given in terms of the energy equivalent if both U.G. 2 and silver filters had been used together, although in most experiments the U.G. 2 filter alone was used and the actual energy received was correspondingly higher (about 20 ×), as explained in § 2 (*b*).

It will be seen that there is much the same toleration to a high dose whether it is divided or not. When the total dose rises much above 2×10^{10} ergs/cm.^{2} the immediate lethal effect is strong, however the dose is administered. Thus, no great increase of irradiation can be tolerated, with the degree of fractionation and the short intervals used here.

It was observed, however, that the treated males showed individual differences in their mortality and mutation rate, for when they were bred in mass cultures and transferred at regular intervals to fresh cultures with new females, the mutation effects seemed to disappear rapidly, as seen in table 2.

TABLE 2. MUTATION RATE IN *y w* DROSOPHILA SURVIVING FOR VARIOUS PERIODS AFTER ULTRA-VIOLET IRRADIATION

	Test for mutations in flies taken immediately after irradiation			Test for mutations in later survivors			
Dose $\times 10^{10}$ ergs/cm.^{2}	No. of viable cultures	No. of lethals	% of lethals	Days after irradiation when parents were transferred to test cultures	No. of viable cultures	No. of lethals	% of lethals
2·0	280	6	2·1	5	313	2	0·6
2·0	493	9	1·8	10	230	0	0·0
1·8–2·0	545	15	2·7	15	128	0	0·0
2·0	213	5	2·4	30	92	0	0·0

At first sight it might appear that the mutation effect in the treated flies declines with time, as in the case of X-rays (Harris 1929; Hanson and Heys 1929), where it was shown to be due to (*a*) a higher mutation rate in mature spermatozoa as compared with the immature germ cells, which are used in later breeding, (*b*) a higher proliferation rate of unmutated as compared with mutated immature cells (Serebrovskaya and Shapiro 1935; Kossikov 1936, 1937). But the drop in mutation frequency appears much sooner in the case of ultra-violet than of X-ray treatment and hence cannot be explained by these causes: it is almost complete in 5 days, whereas the mature spermatozoa are not exhausted until some 10 days after treatment, at 24° C. On the other hand, the curve of mutation rate is closely paralleled by that of the survival of the treated flies, as there was an especially high mortality in the first 5 days, after which the number of survivors remained fairly constant. When the dose exceeded the standard 2×10^{10} ergs/cm.^{2}, nearly all the flies died in the first 5 days, and as our table shows only experiments in which later broods were obtained for comparison with the earlier ones, no dose higher than the standard is shown in it. At these higher levels, however, the mutation rate was higher.

That these deaths were really selective, killing preferentially those individuals in which a higher mutation rate was induced, was then proved by a special experiment, employing stocks to be described in § 2 (*d*). Males were separated at random into two equal portions after irradiation with the 'optimum' dose. The first portion was bred in the usual way, in mass cultures in bottles, and the F_1 females were then bred individually in vials for the detection of sex-linked lethals in F_2. The second portion of treated males was bred individually in vials, one male to two virgin females. After 5 days at 24° C the vials were examined, and only those kept in which the male had died. In only a minority of vials thus selected (some 25 % in the experiment with non-yellow stock) were any offspring produced; these were used for the tests for lethals. Table 3 gives details of such an experiment, in which two stocks, y^2 (moderately pigmented) and non-yellow (normally pigmented), were used.

It is clearly shown that the individuals which had suffered more deleterious effects on their own life were also those in which mutations were more abundant (the mutation rate being some 75 % more in the offspring of the flies which died early than in those not selected). Dismissing the assumption that the rays produce their mutational effects indirectly on the germ cells via the soma, this suggests differences in the penetration of the rays into the individual flies, giving a parallel increase of mutational effect and somatic injury. The question of what factors are more important in causing these differences—amount of pressure on the abdomen, orientation of the fly, variable pigmentation and chitin, size of fly, etc.—remains an open one. The existence of these differences provides, however, a valuable method of increasing the general mutation rate by selecting, in vial cultures, the offspring of those males which die in the first few days after treatment. This was of particular value in the later experiments mentioned in § 2 (*d*).

TABLE 3. COMPARISON OF MASS CULTURES WITH INDIVIDUAL CULTURES SELECTED ON BASIS OF DEGREE OF INJURY TO TREATED PARENT

(Irradiated males crossed to 'all' females described on p. 504. F_1 females of *Hw* type bred individually for detection of lethals in F_2.)

Composition of irradiated males	$y^2\ sc^8\ B\ w^a$	$sc^8\ B\ w^a$
Body colour of irradiated males	Moderate yellow	Non-yellow (normal)
Average dose (in 10^{10} ergs/cm.^{2})	2·0	2·5
Mass cultures (*ca.* 25 males and 50 females per bottle of P_1):		
Bottles of P_1	38	45
F_1 ♀♀ found	927	805
F_1 ♀♀ (*Hw*) tested	438	302
Lethals	13	8
Lethal %	3·0	2·6
Individual cultures (1 male and 2 females per vial of P_1):		
Vials of P_1 used	310	310
Fertile vials from males dying early	86	34
F_1 ♀♀ found in latter	880	220
F_1 ♀♀ (*Hw*) tested	412	113
Lethals	25	5
Lethal %	5·4	4·4

(*b*) *On the spectral region effective for mutation*

In experiments carried out on plant material (Stadler *et al.* 1936, 1938, 1939; Noethling and Stubbe 1934, 1937; Hollaender 1939; Knapp and Schreiber 1939) it has been possible to compare the effectiveness of different wave-lengths, since the small size of the objects (spores and pollen) allows a large number to be exposed to the narrow bands of monochromatic radiation in the conventional spectrograph, without excessive absorption of the light before it reaches the region of the genetic material. With the exception of Stubbe and Noethling, these workers agree in finding that, proceeding from the visible light 'downwards' towards the shorter ultra-violet, the effective region begins at about 313 mμ, the waves in the bands above this being ineffective, and those in the bands between 303 and 280 mμ decidedly effective. Below this again there is some increase in the rate, until a maximum appears to be reached, between 270 and 250 mμ, but the increase is not as rapid as that of the lethal effect, so that it is difficult to get significant data on mutation for very short waves.

In *Drosophila*, Reuss (1935), using all the light transmitted by Uviol glass filters, which are opaque below 280 mμ, obtained, with his highest doses, $6{\cdot}5 \pm 1{\cdot}5$ % of sex-linked lethal mutations, and our results are in conformity with this. Working with whole flies, it is probable that some 99·9 % of the ultra-violet energy from the whole mercury spectrum is absorbed before reaching the germplasm. That the absorption by the chitin rises rapidly with decrease of the wave-length has been shown by Eloff and Bosazza (1938) in tests on thin layers of this material, measured spectrographically, and in tests of the survival of *Drosophila* pupae. McAulay and Taylor (1939) made monochromator tests on *Drosophila* larvae, and showed that the direct lethal dose at 297 mμ is of the order of 10^5 ergs/cm.^{2}, that is, $0{\cdot}5 \times 10^{-5}$ of the dose required by us to produce 3 % of sex-linked lethal mutations in adult *Drosophila* with a mixed radiation of 320–280 mμ. Allowing for the different sensitivity of the material and the factors involved in spectral tests, it is hardly an overestimate to suggest that not more than one-thousandth of the incident energy reaches the gonads and is available for producing genetic effects. This is supported by results of Altenburg (1934), who used whole flies and eggs in the stage of an exposed 'polar cap'; dose for dose, the mutation frequency obtained in the former was less than one-thousandth that in the latter material.

Since monochromatic radiation offers great difficulties with adult *Drosophila*, owing to the twin factors of high absorption and low intensity, the use of filters, as in our early experiments, has been continued. In the first tests, the use of different filters (Schott and Genossen U.G. 2, 1 or 2 mm., and Chance Uviol, 2 mm. thick) suggested that the lower region of the spectrum (that with shorter wave-lengths) was the main effective agent of mutation. In the present series, the use of a filter of 1 mm. of U.G. 2 together with a supplementary filter of silver deposited on quartz has enabled a more definite conclusion to be reached.

The preparation of the accessory silver filter requires special care. A chemically clean quartz plate 5 cm. square and 3 mm. thick is placed in a glass dish, contained in a large beaker of warm water and heated by a 100 W clear glass filament lamp. 10 c.c. of 10 % silver nitrate, made strongly ammoniacal and then brought nearly to neutrality with nitric acid, is poured into the dish and 2 c.c. of 50 % glucose added. The mixture slowly warms up and the deposition of silver is allowed to proceed until visible light from the lamp filament can no longer be seen on looking vertically down from above. The dish is immersed in the bath, and then washed with distilled water till free from silver salts. The quartz plate is lifted out and dried. There should be no deposit of silver on the lower side, as otherwise the result will be unsatisfactory, and the filter will transmit visible light. The silver film is exceedingly thin and delicate, and must be discarded as soon as pinholes appear. (No satisfactory varnish or preservative coating which will transmit ultra-violet freely has been found.) This film has a good cut-off on the long-wave side between 365 and 313 mμ.

Physical tests of this film, combined with the U.G.2 filter using a thermopile and large-aperture spectrograph of moderate dispersion and considerable aberration, gave results showing that the addition of the silver reduced the light transmitted to between 5 and 10 % of that passing the U.G. glass alone. One of these tests (in which the silver film was probably somewhat thinner than usual) is shown below.

TABLE 4. RELATIVE AMOUNTS OF ENERGY OF VARIOUS ULTRA-VIOLET WAVE-LENGTHS TRANSMITTED BY GLASS FILTER U.G. 2 WITH AND WITHOUT ACCESSORY SILVER FILTER

Region (in mμ)	400–320	320–300	300–290	290–280
Principal band	365	313	297	280
U.G. 2 filter only	16·5	1·0	0·6	*
U.G. 2 + silver filter	1·1	0·87	0·9?	*

* Less than 0·01, the lowest measurable amount.

Overlapping of the wave-length regions, which undoubtedly occurred, would tend to smooth the differences observed between such regions, so that the differences are, if anything, greater than those shown. The absolute values are arbitrary, that for the region 320–300 mμ being denominated 1; most of this was due to the 313 mμ line. In the region 300–280 mμ, the energy is almost all from λ 297 mμ. The region 290–280 mμ represents an extremely small fraction, whose effect is probably negligible when the effect of selective absorption in the chitin is considered. The 280 mμ line is distinguishable only by its fluorescent effect and not measurable by physical means, owing to the practically complete opacity of the U.G. 2 filter. (It is, of course, a very powerful component of the unfiltered radiation from the pressure mercury arc.) No waves below 280 mμ are transmitted. In the region 400–320 mμ, more than 90 % of the energy passed by the U.G. 2

filter is from λ 365 mμ, the total for that part of the region which is below this being about 7 %. The silver filter, by cutting out the 365 mμ line, may be said virtually to narrow the effective region to between 320 and 290 mμ; in so doing, it reduces the intensity in this region by only some 15 %.

Biophysically, this silver filter was tested by exposing *Drosophila* of a moderate-yellow apricot-eyed stock ($y^2\ sc^8\ B\ w^a$) to the radiation transmitted by this filter in series with the U.G. 2, while for comparison, an equal number (fifty at a time) were exposed for the same time and under the same conditions to the radiation from the U.G. 2 filter only, which was between 10 and 20 times greater in amount. The results are given in table 5.

The mutation rates appear identical despite there being some tenfold difference here in the total radiant energy. It may be concluded with confidence that the region above 320 mμ has less than 5 % as great an efficiency in producing mutations as the region below (320–290 mμ). Thus, with the pressure mercury arc, the upper limit of mutation effect in the ultra-violet may be taken as near 320 mμ, and the effect must begin between this and 300 mμ. But the region of maximum efficiency, and the curve relating mutation frequency to wave-length, are not deducible from these data. It is probable that this must await a time when direct irradiation of the germ cells can be carried out efficiently. Mastery of the technique of artificial insemination would be invaluable in this connexion, but so far the attempts made in this direction have not been sufficiently successful to allow the use of this method in such experiments.

(c) *Combination of gene mutation and translocation test*

The experiments previously described (Muller and Mackenzie 1939) involved separate tests for mutations and translocations, and left uncertain the individual response to dosage which might result in unequal response in the two parts of the experiment; this could only be partly corrected by the use of considerable numbers. To eliminate this objection, a genetic mechanism was elaborated so that the mutations and translocations could be detected in the offspring from the same cross.

The males used for irradiation contained in the *X*-chromosome the genes for yellow (*y*) and white (*w*), which reduce pigmentation, and a long inversion combination (sc^{4L}, sc^{8R}) to reduce the occurrence of cross-overs. These were mated to females containing a shorter inversion (δ 49) and other markers (*sc v f*) in the *X*, and markers in the second (*dp*, dumpy wings) and third (*e*, ebony body colour) chromosomes, respectively. The F_1 females from this cross gave practically no cross-over offspring (F_2), so

TABLE 5. RELATIVE EFFECTIVENESS OF LIGHT OF REGIONS (*a*) 320–280 mμ AND (*b*) 400–280 mμ IN MUTATION PRODUCTION

	(*a*)					(*b*)					
No. of experimental series	Total energy (in 10^{10} ergs/cm.^{2})	No. of flies dead after 3 days	No. of fertile cultures of F_1 (n_a)	No. of lethals found (P_a)	% lethals (p_a)	Total energy (in 10^{10} ergs/cm.^{2})	No. of flies dead after 3 days	No. of fertile cultures of F_1 (n_b)	No. of lethals found (P_b)	% lethals (p_b)	Wt. of series n_h* (harmonic mean of n_a and n_b)
1	2·4	12	101	5	4·9	42	15	172	8	4·7	126
2	1·9	17	128	3	2·3	36	8	38	0	0·0	51
3	1·8	11	63	2	3·2	38	13	138	4	2·9	86
4	2·0	9	88	3	3·4	44	24	165	8	4·8	114
Sum (Σ) or weighted average (av.)	2·1 (av.)	49 (Σ)	380 (Σ)	13 (Σ)	3·7 (av.)	40·9 (av.)	60 (Σ)	513 (Σ)	20 (Σ)	3·7 (av.)	377 (Σ)

Difference (%) = $3{\cdot}7 - 3{\cdot}7 = 0{\cdot}0 \pm 0{\cdot}9$.

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* The use of this system of weighting is explained in a parallel paper (Muller 1940). The standard error of this difference has the value $\sqrt{[\Sigma(n_h pq)]}/\Sigma n_h$, where p represents the proportion of lethals found in a given series with both spectral regions taken together (e.g. for series 1, $p = (5+8)/(101+172)$ and $q = 1-p$).

that the F_2 derived from the F_1 females could readily be used for detecting sex-linked lethal mutations. Such mutations were indicated by the absence of *y w* male offspring. At the same time the F_1 males, heterozygous for *dp* and *e*, were crossed to *dp e* females. The progeny (F_2) from this cross gave a test for translocations between the *Y*, II and III chromosomes. *Such translocations would be indicated by the absence of recombination* between the characters for sex, wing shape, and body colour.

In this experiment, chiefly owing to the improved technique previously described, a higher mutation rate was obtained than in previous work. The results are given in table 6, together with the previous results for comparison.

TABLE 6. SIMULTANEOUS TESTS FOR GENE MUTATIONS (SEX-LINKED LETHALS) AND TRANSLOCATIONS

			Lethal test			Translocation test		
	Stock	Dose ($\times 10^{10}$ ergs/cm.^{2})	Cultures	Lethals	%	Cultures	Translocations	%
Earlier experiment* (non-simultaneous tests)	*y w*	1·5–3·0	1676	42	2·57	3425	0	0·0
Present experiment* (simultaneous tests)	$y\ sc^4\ w\ sc^6$	2·0–3·0	1375	64	4·65	1617	0	0·0

* In the earlier experiments, which include the results reported in our earlier communication, *different P_1 males had been used to provide the material for the lethal and translocation tests,* respectively, whereas in the present experiments the same treated P_1 males provided offspring for both tests, as explained in the text.

The complete absence of translocations in this case is striking. The ultra-violet radiation used, producing some 4·3 % of sex-linked lethals, is equivalent to an X-ray dose of 1300 r. or more, since 1 % is produced by 300 r. and the frequency is proportional to dosage. At this dose the percentage of translocations of type II–III alone is at least 2 %; hence the number of these translocations that would have been obtained among 1617 cultures with this dose of X-rays is 40 or more. This number is a minimum, since the assumption is made that the doses were constant, whereas they were in actuality averages: *since the translocations increase more rapidly than the* dose, the result might well have been higher. For the earlier results, on the basis of the 2449 cultures then available, it had been calculated (Muller and Mackenzie 1939) that between 12 and 26 II–III translocations would have been produced, if X-rays of the same gene-mutation producing strength had been used. On the basis of the number 3425, and with the more exact knowledge of translocation production by X-rays now available, the expected number becomes at least 25, for all the earlier cultures taken together, and 25 + 40, or 65 translocations expected in the work as a whole, had X-rays been used. The difference between the X-ray and the ultra-violet effect is therefore definite and significant.

A further point significant in this connexion (concerning deletions, which form another group of gross rearrangements akin to translocations) will be mentioned in the next section, dealing principally with minute rearrangements.

(*d*) *Failure of ultra-violet radiation to produce minute rearrangements*

It was established some years ago that minute rearrangements are produced by X-rays, and may be of such small size as to be indistinguishable, cytologically and genetically, from gene mutations (Muller, Prokofyeva and Raffel 1935; Muller 1937 et seq.), and it was further found that their frequency followed the same direct proportionality law as that for gene mutations, thus differing markedly from the gross rearrangements (Belgovsky and Muller 1938; Belgovsky 1939; Muller, Makki and Sidky 1938; Muller 1938, 1939, 1940). It remained doubtful, therefore, whether there was any clear-cut difference between minute rearrangements and gene mutations. If there was not, gene mutations were only extremely minute rearrangements of units smaller than genes (as these had been conceived of); the distinction between 'intra-genic' and 'inter-genic' vanished, and the gene was no longer a body having definite limits, but merely a region of the chromosome 'conceptually isolated' for convenience of reference (see Muller 1937, 1938; Goldschmidt 1937, 1938, 1939).

With this problem in view, the discriminatory action of ultra-violet in producing gene mutations but not gross rearrangements made it desirable to seek whether the distinction could be pushed still further, so as now to differentiate between gene mutations and minute rearrangements. Accordingly, a large-scale experiment was planned, providing a genetic arrangement in which such changes could be ascertained by inspection, and depending upon the fact that such rearrangements are unusually numerous in or near heterochromatic regions. Thus if a stock is used in which, by a previous gross rearrangement, the normal allele of a known visible recessive mutant has been placed near a heterochromatic region, there often appear minute rearrangements having the recessive mutant phenotype, combined with a recessive lethal effect (Muller, Raffel, Gershenson and Prokofyeva-Belgovskaya 1937; Muller, Prokofyeva-Belgovskaya and Raffel 1937; Belgovsky and Muller 1937; Raffel 1938; Belgovsky 1938).

A genetic arrangement of this type occurs in scute-8, where by a long inversion of the X a part of the heterochromatic region that had been near the centromere has been transferred to the opposite end, adjoining the loci of yellow and achaete. When crossed to yellow, even without X-raying, such flies give offspring containing a considerably higher frequency of yellow mutants than would be found in an ordinary cross of non-yellow with yellow. Tests show that the yellows derived from scute-8 are usually lethal when homozygous and involve a minute rearrangement (possibly a deletion) in this region. As a result of X-raying, minute rearrangements of this kind are produced in much larger numbers, and their abundance is much greater than that of mutants of any of the 'visible loci' lying in the euchromatic parts of the chromosome.

The most frequently occurring minute rearrangements of euchromatic regions that can be recognized by inspection are the minute deletions giving rise to the dominant Notch wing, by change from the normal non-notched condition, and the minute deletions and inversions giving rise to the normal non-bar type of eye by change from the minute duplication responsible for the dominant 'Bar' character. It was planned to look for these changes as well as for those involving the yellow locus in scute-8. This required the use of scute-8 flies normal with regard to Notch but containing Bar, and not having the extreme allele of yellow. Accordingly, a scute-8 stock of rather light body colour, containing a moderate allele of yellow (y^2) combined with apricot (w^a) and dominant Bar (B) eyes, was built up (formula: $y^2\ sc^8\ B\ w^a$). Since in the course of the work with this stock it was found too time-consuming to discriminate between the yellow mutants of the extreme type (y) and the ordinary flies of this stock (y^2), a second stock with the normal allele of yellow ($sc^8\ B\ w^a$) was later used.

Males of either type, after irradiation, were crossed to females homozygous for yellow, of a stock named 'all', which contained a large group of recessive mutants (mostly heterozygous) in the X, designed specially for the detection of mutations in various specific loci. The complete formula of the X was usually $\frac{y\ ac\ \ sc\ pn\ w\ \ \ v\ \ \ g\ f}{y\ Hw\ \ \ \ \ \ \ \ \ \ w\ \delta 49\ lz^s\ f}$, but flies homozygous for either of the differing chromosomes were also present. Those having both chromosomes as in the lower line are practically sterile, but the type homozygous for the upper line were also used in the cross.

The chief recessive mutants arising in the F_1 of this cross would be y, w, f, and reversions of Bar to normal eye. The females were carefully examined for these changes, as well as for Notch wings. As the rather variable w^a/w eye colour of the offspring renders them unsuitable for the detection of eye-colour mutations other than white they were not checked for these; in the same way, the *sc* and *ac* bristle characters were too variable in presence of sc^8 to be used as criteria. Cubitus interruptus (*ci*) in the fourth chromosome was present homozygously in the all females, but it only develops satisfactorily in cultures at low temperature, which is not a convenient condition in our work.

The F_1 females are of two types, heterozygous for the upper or lower part of the 'all' formula shown above; both types are used for the detection of minute rearrangements by inspection of the body, wings and eyes, and those containing the dominant *Hw* character are also well adapted for the determination of sex-linked lethals by breeding tests, when individual cultures of them are made. For they contain not only heterozygous markers but also the two inversions sc^8 and $\delta 49$, which together prevent crossovers from appearing in the next generation. A lethal is thus detected by the absence of sc^8 B w^a males among the offspring. The majority of these F_1 *Hw* females were bred individually in vials to determine the efficacy of the ultra-violet radiation in the P_1 males. Table 7 gives a summary of the results obtained with some 11,000 F_1 females derived from the crosses of the y^2 and normally pigmented stocks of sc^8, treated according to the selective mortality scheme.

Among the F_1 females noted in the table, seven gynandromorphs are included, and there was one mosaic apparently a male but possibly also a gynandromorph. This number was not in excess of that which may be found in an untreated group of similar size.

It is noteworthy that no mutation in the specific loci was found, despite a fairly high mutation frequency of sex-linked lethals, which corresponds closely to an X-ray dose of 1000 r. This correspondence enables this experiment to be compared directly with one of Muller and Makki, as shown in table 7. While the non-appearance of *w* and *f* may well be accidental, the absence of any *y*, *B* or *N*, which, collectively, were expected in considerably higher frequency, on the basis of the X-ray results, is much more conspicuous. In fact, statistical treatment shows that the difference between the ultra-violet and the X-ray experiment with respect to the frequencies of minute rearrangements found is significant. If, then, we may take these results at their face value, we shall be led to the conclusion that ultra-violet rays cannot produce minute rearrangements, or that, at least, they are far less efficacious in this respect than X-rays, when doses that are equivalent in their power of producing gene mutations are considered. At the same

TABLE 7. EXPERIMENT FOR THE DETECTION OF MINUTE REARRANGEMENTS AND OTHER MUTATIONS INVOLVING SPECIFIC LOCI (y, w, N, f, B). IRRADIATED SCUTE-8 MALES CROSSED WITH ALL FEMALES

Type of treatment	Composition of treated male	Vials of P_1 made up	Fertile vials from males dying early	No. of F_1 females from latter	F_1 females (Hw) tested for lethals	No. of lethals found	% lethals	Visible mutations			F_1 males with deleted X's (non-yellow)
								To yellow	To N or B^+	To w or f	
Ultra-violet	$y^3\ sc^8\ B\ w^a$	2515	599	7,236	3266	129	3·9	0	0	0	0
Ultra-violet	$sc^8\ B\ w^a$	1415	241	3,755	1790	53	3·0	0	0	0	0
Ultra-violet	Sum of above	3920	840	10,991	5056	182	3·6	0	0	0	0
X-rays* 1000 r.	$sc^8\ B\ w^a$	—	—	11,000	—	—	3·6	11	5	3	2
Controls	$sc^8\ B\ w^a$	—	—	11,000	—	—	0·1–1·0	1–	0	1–	0

* The numbers of visible mutations shown for 1000 r. and for the controls are those expected for a total count of 11,000 F_1 females as calculated from the results of Muller and Makki (see Muller 1940). The percentages of lethals given for 1000 r. and the controls are based on all previous work, the percentages of lethals expected after 1000 r. X-rays being 3·3 % + the control frequency; the latter may vary between 0·1 and 1·0 %, as noted above, but the most common frequency is about 0·3 %, as here assumed.

time, it should be recognized that these results are preliminary, and the conclusion provisional. It will be desirable to obtain more data of this kind, involving the treatment of non-yellow flies exclusively (because of the greater certainty of distinguishing between non-yellow and extreme yellow than between moderate yellow and extreme yellow) before regarding the point in question as definitely proved.

The absence of yellow (*y*) exceptions gives evidence regarding a further point. It indicates the impossibility or difficulty of producing 'terminal deficiencies' in *Drosophila* by ultra-violet light, of the kind found by Stadler and by McClintock to be produced in maize by both ultra-violet light and X-rays. If a 'simple break' took place in the especially breakable heterochromatic region of the scute-8 chromosome just to the right of *y* and *ac* and were followed by a 'healing' of the broken end of the proximal fragment (i.e. by change of the originally interstitial gene at the broken end into a gene with the properties of a telomere), then the proximal fragment would be able to survive and give rise to a yellow exception resembling the exceptions which are formed through minute rearrangement as a result of X-raying. Since these exceptions have not been obtained we must conclude that neither minute rearrangement nor simple breakage associated with healing has taken place, at least not with sufficient abundance for it to have been found in counts of the given size. This is in agreement with the evidence from other sources that the change of gene polarity required for such a process—no matter whether or not we classify it as a gene mutation—is far less easily brought about in *Drosophila* than in the plant material studied with reference to this point.

It may also be noted that no males bearing grossly deleted *X*-chromosomes were produced in the above experiment. As only two would have been expected from X-rays, this result is not in itself significant, but it is seen to be in line with the already existing evidence against the production of gross rearrangements, which has hitherto concerned itself only with the other two of the three most commonly found types (i.e. with translocations and inversions). This then adds to the strength of the earlier conclusion, that breakage of the type *not* followed by healing of the broken end (and therefore of a kind capable of leading to gross rearrangements of gene order) is not produced by ultra-violet.

DISCUSSION

The finding that the effective wave-lengths begin in the region between λ 320 and λ 300 mμ agrees with the results of the work on plant material and with the idea that it is absorption by the nucleic acid which is responsible for most or practically all of the mutation effects obtained from the ultra-violet irradiation used. This interpretation is not yet definitely proved, since protein and a few other cellular materials begin to absorb in the same region. However, spermatozoa contain little which absorbs ultra-violet light to any noteworthy degree in the region about λ 300 mμ other than nucleic acid and protein and, as Caspersson has pointed out (1936), the absorption of these wave-lengths by the nucleic acid is considerably greater than that by the protein. Although we have not been able to adduce the additional evidence, gained in the plant material, that the mutational effectiveness rises to a maximum somewhere between λ 265 and λ 250 mμ and then begins to decline, like the absorption by nucleic acid and unlike that by protein and most amino-acids, nevertheless, the above agreement in respect of the region where the effect begins, taken together with the similar composition and reactivity of animal and plant chromosomes, makes it extremely probable that in our material, too, it is the nucleic acid which is the chief absorptive agent for the mutation effects observed.

It will be of interest to study the energetics of the mutational reaction from the point of view of the efficacy of the absorbed quanta of ultra-violet radiation. That is, what is the proportion of absorbed quanta that are 'successful' in the sense of giving rise to a mutation that can be detected, and what relation does this proportion bear to the dosage conditions, i.e. to the space and time distribution of these quanta? The comparison of these results with the corresponding ones already obtained for the success of ionizations produced by X and similar rays would be especially instructive. Such figures cannot be adduced, however, until it has become possible to measure far more accurately what part of the impinging radiation penetrates the body layers and reaches the spermatozoa, as well as how much the absorption by an individual spermatozoon is. Caspersson has solved the latter problem for other cells by application of a micrometric method which could be used without essential modification for spermatozoa, and perhaps a similar set-up could be used for determining the amount of absorption by the body wall. However, a more direct attack, allowing a better comparison both of the absorptional and of the mutational effectiveness of different wave-lengths, could be made if the germ cells could be irradiated free from any screening by the interposed body wall and chitinous material that is unavoidable when whole flies are treated. A further development of artificial insemination technique would afford a way out of this difficulty. Altenburg's polar-cap method might also have possibilities in this connexion, although it is difficult by this method to obtain such large numbers of separate mutations as an adequate statistical treatment would require. Another possibility would be irradiation of the excised gonads, followed by their implantation. All this must be left for future studies.

Even before such studies are made, certain further conclusions concerning the mutational reaction would follow, if we accept the inference that the nucleic acid constitutes the primary site of ultra-violet absorption for the mutational reaction. For the composition of nucleic acid is too constant to permit of its being, by itself, the material in which the differences characterizing one gene as distinct from another reside. Moreover, it is well known that the amount of nucleic acid in a given chromosome varies greatly in the cycles of cell growth and division. Hence the nucleic acid cannot be regarded as more than accessory to the distinctive gene substances.

The distinctive gene substances then are probably of protein nature, although by far the greater bulk of the protein present in spermatozoa must be non-genic, as shown by the great differences in type of spermatozoan protein in not distantly related species (see review by Gulick 1938). It might, indeed, have been postulated, in view of the latter finding, that the genes were not protein at all but had their basis in the very small amount of other material present in spermatozoa. However, the lack of other material than nucleo-protein in viruses, despite their having gene properties, indicates that the protein must be the essential substance, and this is supported by general considerations of the possibilities of protein, as compared with other substances, in respect to diversification of composition and of reaction, relationship to enzymes, including the protein-forming enzymes themselves (proteases), and the resultant greater likelihood of proteins having the property of autosynthesis.

Thus we reach the conclusion that although the nucleic acid absorbs the ultra-violet quanta which precipitate the occurrence of a gene mutation, the protein must somehow participate in the mutational reaction itself. How tightly and directly bound the nucleic acid in question is to this protein we do not know, nor whether the nucleic acid groups serve as connecting links in the individualized protein pattern characterizing the given gene. But in any case some of the mutational changes of this pattern would require alterations in the connexions of the protein component. Thus there would have to be some sort of transference of energy (that is to say, a further reaction) from the activated nucleic acid to the protein—presumably, to the protein that is in its immediate neighbourhood—before the mutation could take place, and to this extent the mutation would be an indirect result of the irradiation. It would be premature to speculate upon how direct this transference of energy may be and whether it may also involve a release of energy; that is, we are justified in saying only that a secondary reaction, in the protein component of the nucleo-protein, occurs as a result of the change first induced in the nucleic acid component. This is not to say that absorption of a quantum directly by the protein could not result in a mutation, but only that, with the wave-lengths and intensities here used, the mutations actually obtained usually result from quantum absorptions by the nucleic acid, with resultant secondary changes in the protein, the absorption by the protein itself occurring too rarely, under these experimental conditions, to play an important role in the results obtained.

If it is true that the ultra-violet-induced gene mutations are usually secondary effects, in the above sense, then the stricture (Muller 1932, 1937, 1940) against the use of the frequency of radiation-induced mutations as a direct measure of gene size and number receives further support. So long as it was assumed (Blackwood 1932; Gowen and Gay 1933; Haskins 1935 et seq.; Marshak 1935 et seq.; Timoféeff-Ressovsky and Zimmer 1935; Lea 1940) that mutation was induced by radiation only when the atom originally activated lay within the gene itself, then the number of such atoms in a given gene could be calculated from the frequency of given mutations, occurring in response to given doses of radiation. But once it is admitted that the activation may be initiated outside the gene, with only a transferred or secondary effect upon the latter, this method of calculation can no longer be employed for this purpose. In the previous criticisms by Muller (1932, 1937, 1940) the experimental evidence for an indirect effect was derived entirely from results concerning structural changes (rearrangements). The work herein discussed now allows us to carry the argument further, basing it upon the evidence concerning gene mutations themselves. At the same time it should be recognized that the method in question, although incapable of telling the size of the gene itself, may give results of interest concerning that material whose atoms, when activated, can react in such a way that, secondarily, mutation of the gene is induced.

The finding that gross rearrangements of chromosomes are not produced in appreciable numbers by ultra-violet light must, as Stadler (1939) has pointed out, not be regarded as a proof of the absolute negative that no gross rearrangements can be produced in this way. It does mean, however, that the ultra-violet-induced activations, as compared with the X-ray-induced ones, are distinctly discriminatory, in that a group of the former capable of producing a given number of gene mutations would produce far fewer (if any) gross rearrangements, than a group of the latter capable of producing the same number of gene mutations. It is this difference in the preferential mode of action that next requires consideration, in the light of what is known concerning the way in which gross rearrangements come about.

Previous studies have shown that the mechanism of production of gross rearrangements, whether in animal (Muller 1938–40) or plant (Sax 1938, 1939) material, is by breakage of the chromosome thread at two or more points, leaving the broken ends adhesive, followed sooner or later by joining together of the broken ends in a new order. The different breaks are produced independently of one another, by separate individual ionizations or other activations, the number of primary breaks being linearly proportional to the number of induced activations (including ionizations). Even chromosomes broken by mechanical means have their broken ends thus adhesive and capable of forming new combinations (McClintock 1938, 1939). Hence, in the production of gross rearrangements, breakage of the chromonema is the primary step. It thus seems very probable that breakage of the chromonema—at least of the thoroughgoing type, resembling mechanical breakage, which permits the formation of gross rearrangements—cannot be produced by that kind of activation of the nucleic acid which can give rise to gene mutations. Moreover, escape may not be had from this conclusion by erecting the special hypothesis that the breakage is produced by ultra-violet, but that the broken end so produced becomes 'healed' and incapable of union with other pieces. For, as we have seen in the last section, there is experimental evidence against the 'terminal deficiencies', that would result from such a process, being produced by ultra-violet.

We have then a certain basis for distinction between the mechanism giving rise to 'thoroughgoing' chromonemal breaks and to gene mutations. Either the chromonemal breaks capable of leading to gross rearrangements require some other kind of activation of nucleic acid than that which ultra-violet can induce (see paragraph below) or they require the activation of some other material than the nucleic acid, material which the X-rays but not the ultra-violet rays are capable of affecting in sufficient abundance to give us the gross rearrangement effects observed after X-ray treatment. It is well known that the ionizations and other activations produced by X-rays and rays of still higher energy, such as γ-rays, are much more indiscriminate in respect of the site at which they occur than the activations produced by ultra-violet rays. Whereas the latter, at the wave-lengths and doses used, activate little but the nucleic acid, the X and higher energy rays ionize and otherwise activate the water and all the other cell constituents in a largely random manner. As water is by far the most abundant substance in the cell it may well be (as suggested by Delbrück in a personal communication, and as independently proposed by Fricke and Demerec 1937) that it is usually the ionization of the water which constitutes the primary change in the X-ray effect, and that following this there is a transfer of energy, that is, a further reaction, which in turn affects the chromonema or gene. It may, however, be the protein or some other constituent in which the primary effect of the X-rays that results in breakage usually resides. Whatever it is, the effect of the X-rays too, in breaking the chromonema, is often a transferred effect, as shown by the frequent breakage of the same thread at two nearby points by a single ionization, giving a minute rearrangement.

It is further to be observed that a large proportion of the atomic changes produced in the track of the electrons released by X and γ irradiation are not low-energy activations, involving merely the removal of a valence electron to a more distant atomic orbit, but actual ionizations, which involve the expulsion of an electron. (Often this is not the valence electron; but the final effect will usually be the transferred loss of the valence electron.) And these ionizations occur in largely unselected atoms (with a likelihood that is a simple function of their mass), whereas the ultra-violet activations are much more selective, involving given atoms in given molecules. Finally, the ionizations and other activations induced by the ultra-violet tend to occur singly (being randomly distributed in space, with regard to the sensitive substance), whereas those induced by X-rays tend to occur in clusters. All these factors might produce a basis for a difference in the effect of the two agents, even if the primary change occurred in the same substance.

It is the above-mentioned greater selectivity of the ultra-violet radiation, in all the ways above mentioned (type of molecule affected, atom affected, electron affected, and degree of activation by one quantum), that offers the hope that more insight into the nature of the gene-mutational reaction may eventually be gained by this means than by the X-ray method. This would, however, require much more information than is now available concerning the chemical details of the changes which ultra-violet may induce in nucleic acid and, secondarily, in substances chemically associated with the latter.

The above discussion has concerned itself with the problem of the difference in mechanism of production of gross rearrangements and gene mutations. So long as it was conceivable that the production of minute rearrangements followed different principles from that of gross ones, in response to ultra-violet irradiation, it might more readily be maintained that the results concerning gross rearrangements should not be generalized, and that ultra-violet after all might produce chromonemal breakage, but of a specialized type resulting in minuter changes. This in turn would help to obliterate the supposed distinction between minute rearrangements and gene mutations.

Thus it remained to be determined whether or not the minute rearrangements were essentially similar to the gross rearrangements in their method of production. Our present results with the ultra-violet, indicating that minute rearrangements are not produced by it—at least not in the abundance to have been expected from the same gene-mutational strength of X and higher-energy radiation—points to the existence of an essential similarity in the mechanism of production of the gross and minute rearrangements. The conception of minute rearrangements as resulting from chromosome breaks like those which produce the gross rearrangements (Muller 1935; Muller, Prokofyeva and Raffel 1935; Muller 1940) thereby receives further support, and it is seen that the gene mutations, on the other hand, are distinct, in their mechanism of production, from both the gross and the minute rearrangements. They can probably be produced by the nucleic acid activation brought about by ultra-violet, while breakages giving demonstrable linear rearrangements of any sort, large or small, cannot be so produced, unless with very considerably more difficulty.

This conclusion is of importance in relation to the current question (see p. 503) as to whether gene mutations may not after all be merely ultra-minute rearrangements of essentially the same kind as the changes large enough to be demonstrable as minute rearrangements. If this view were to be maintained in the face of evidence that demonstrable minute rearrangements are not produced by ultra-violet, some further hypothesis would have to be set up—such as that the two or more breaks presumably produced by an ultra-violet activation, destined to take part in the making of the ultra-minute rearrangement, were necessarily much nearer together than the two induced by a given X-ray ionization. They would in fact have to be so near together, ordinarily, as not to lie in 'separate genes'—that is, in portions of the chromonema so far apart and so different from one another as to be distinguished as separate genes by previous methods. Such an assumption, while not impossible, would seem rather special and arbitrary. Moreover, even if this were true it would not necessarily follow that the genes themselves were not sharply delimited from one another, and the links between the different genes might still be of a different nature than those between their parts, and might be subject to different principles of breakage and reunion. These are questions which still require considerably more study. In the meantime, however, the evidence that the changes definitely demonstrable as *inter*genic (according to the criteria customarily applied) cannot be produced, or only with great difficulty, by an agent—ultra-violet—which produces *intra*genic changes (as measured by the same criteria) with comparative readiness raises a strong presumption that the difference between 'inter-' and 'intra-' genic, as gauged by these criteria, does have a factual basis, in a segmental structure of the genetic material.

The present work suggests a possible means of throwing light on an interesting question concerned with the mutation theory of the origination of cancers. It had been suggested by Boveri (1914) that cancer might arise as a genetic change in a somatic cell, but he conceived this change in terms of the chromosome mechanism more familiar to him at that time, as involving a loss or addition of one or more whole chromosomes, caused by some mitotic abnormality. The more modern genetic viewpoint suggests that if cancer does arise by 'mutation'—a contingency which seems very plausible for some cases of cancer at least, despite the demonstration that others are caused by viruses (see discussion by Auerbach 1940)—the mutation in question is much more likely to be a structural chromosome change or gene mutation than 'aneuploidy' of the whole-chromosome type which Boveri had in mind. This is because (1) the number of different kinds of whole-chromosome changes is far more limited than of structural changes or gene mutations, and hence has less likelihood of being of just the right kind to give rise to a cancerous growth, and because (2) the whole-chromosome changes usually involve such an extreme 'unbalance' of genes as severely to handicap the cells containing them in their metabolic and proliferative activities.

While the non-existence of a whole-chromosome aberration might in favourable instances be revealed by cytological study (since it would be expected to characterize the given cancer tissue as a whole) the question of whether the presumed mutation was of the structural change or gene-mutation type could seldom be settled in this way. Theoretically, gene mutation would provide the likeliest interpretation, for the same arguments which inclined us to regard whole-chromosome change as a less likely cause than either structural change or gene mutation would also incline us to regard the former phenomenon as a less likely cause than the latter. But to get actual experimental evidence for this view would at first sight seem very difficult if not impossible. For such structural changes are often invisible in cytological preparations of mitotic chromosomes, and as cancer tissue is somatic it cannot be subjected to the usual genetic analyses. However, the discriminatory action of ultra-violet, herein dealt with, in producing gene mutations without structural changes might provide a method for throwing light upon the problem.

This method would involve the reputed connexion between ultra-violet irradiation and cancer. It has now and again been claimed that carcinomas may be produced as a result, direct or indirect, of the action of ultra-violet light upon the epidermis, especially in forms with little skin pigmentation. It would be worth while to conduct a series of more exact investigations into this matter, determining how regular is the effect, if any, under given conditions of treatment, and whether the rays themselves actually penetrate to the cells in which the cancer arises. If it could be shown that the curve relating the frequency of the effect to the wave-length of the impinging radiation has its beginning and its maximum in the same regions as the curve for the absorption of ultra-violet light by nucleic acid and for the mutational effectiveness of ultra-violet, when due allowance for the amount of penetration of the different wave-lengths to the tissue in question has been made, then a strong *prima facie* case would be set up for the effect being a mutational one. And not only that, but the conclusion would also be favoured that the mutations giving rise to these cancers were of the gene-mutational type rather than either structural changes in chromosomes or whole-chromosome changes.

It is realized that such work, whether carried out *in vivo* or *in vitro* (the latter by irradiation of tissue cultures, followed by implantation tests), would offer considerable technical difficulties, and require the co-operation of different types of workers on a large scale, but in view of the importance of the problem it would seem very desirable to have such studies made.

For purposes of comparison with such results with ultra-violet light, it would also be desirable to have more exact experimental studies made of the incidence of cancers of various types in animals subjected to irradiation by X-rays. This irradiation should be carried out with doses of different intensities and degrees of fractionation, so that it may be determined what correlation, if any, exists between the incidence of the cancers and the incidence of the different types of genetic effects (gene mutations and structural changes) that have recently been shown to be affected differentially by such variations in treatment.

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The effects of low temperature and acclimatization on the respiratory activity and survival of ram spermatozoa

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The respiratory activity of ram spermatozoa was measured before and after experimental treatment at low temperature.

Sudden cooling has a harmful effect (temperature shock) on the subsequent respiratory activity of the spermatozoa. Temperature shock increases as the temperature is lowered. Temperature shock increases with the time of exposure to the lower temperature. When semen is cooled gradually in stages of 5° C and with an interval between stages, acclimatization of the spermatozoon increases with the length of the interval. If the interval between stages is 2 hr., the semen can be cooled to 1° C and stored at this temperature for 24 hr. without appreciably affecting the subsequent respiratory activity. Rapid warming has no harmful effect upon the subsequent respiratory activity of the spermatozoa.

Temperature shock and acclimatization are factors which affect the apparent optimum temperature for the storage of spermatozoa. With rapid cooling the optimum is higher than with slow cooling. Semen cooled gradually to 1° C and stored at this temperature for 6 days retained 74 % of its initial respiratory activity.

Introduction

The effect of temperature on the mammalian spermatozoon presents many features of interest. It is well known that spermatogenesis ceases if the testis is returned to the body cavity or if it is artificially heated. The effect of this slight rise in temperature is less immediately harmful to the mature spermatozoa, but Knaus (1932) has shown that, in comparison with the duration of fertilizing capacity of spermatozoa in the scrotal isolated epididymis, which is about 40 days in the rabbit, the duration of fertilizing capacity of spermatozoa in the cryptorchid isolated testis is about 7 days, and in the cryptorchid isolated epididymis about 4 days. It may therefore be significant that, in mammals, storage of the spermatozoa takes place in the scrotal epididymis and not in the body cavity where the temperature is higher. Outside the body (*in vitro*), conditions are of course different but the spermatozoa show very great sensitivity to high temperatures. At body temperature they retain fertilizing capacity for about 10 hr. only (Walton 1930). At 45° C they are almost instantaneously destroyed. As the temperature of storage is reduced, the time of survival increases. This increase in survival runs parallel with a decrease in the activity of the cell, and it is probable that the increased survival is due to a reduction in the rates of those metabolic processes which lead ultimately to exhaustion or destruction of the cell.

This effect of lowered temperature on survival has been noted by many workers. Walton (1930) found, however, that there was an optimum temperature of storage at about 12° C, and below this some unfavourable reaction set in which was more marked as the temperature of 0° C was reached. Various authors have confirmed the existence of an optimum between 15 and 0° C, but are not agreed as to its exact position on the temperature scale. This disagreement is probably explained by the results recorded in this paper. Milovanov (1934) found that sudden cooling had a deleterious effect, which he termed 'temperature shock', on spermatozoa; and in consequence all rooms used for the collection of semen for artificial insemination in the U.S.S.R. are specially heated. Birillo and Puhaljaskii (1936) recommend also the use of a vacuum cup for the collection of bull semen and, for storage, slow cooling at a rate of about 10° C per hour. This present paper is an attempt to study temperature shock on a more quantitative basis, using as a criterion of its effect the respiratory activity of the cell.

In order to assess activity of the spermatozoon, most workers have adopted a system of arbitrary gradings of activity according to the appearance of a drop of semen under the microscope. This method, although of some value, can give only approximate results, since it is semi-quantitative and largely subjective. Measurement of the respiratory activity is employed in this investigation because it is both quantitative and objective.

Furthermore, Walton and Edwards (1938) and Walton (1939) have shown that the respiratory activity can be used as a criterion of sperm activity which is closely correlated with the fertility of the male.

METHODS

Semen taken from Suffolk rams during a 9 months period from October 1939 to July 1940 was used. The semen was collected in an artificial vagina by the method described in detail by Walton (1938). In the present investigation a vacuum cup was used in place of the usual receiver for the semen. Before use the cup was disinfected with 65 % alcohol, dried in an oven and kept before use in a warm chamber. As the temperature in the artificial vagina during collection is maintained at about 40–45° C, the temperature (verified by thermocouples) inside the cup is usually about 35° C and never below 30° C. Thus the semen is not subjected to any temperature shock during collection. An ewe, which need not be in oestrus, was used in the service crate. When the ram was brought up to the ewe, he mounted for service and his penis was directed into the artificial vagina. He ejaculated within a few seconds. As soon as the semen was obtained it was removed to a warm chamber (25–30° C) in the laboratory. As one ejaculate of the ram is seldom more than about 1 c.c., we collected two samples with an interval of about 7 min. between each collection. The two samples were mixed thoroughly together before use in the experiments. All manipulation of the semen was carried out in the warm chamber. Sterilized pyrex glass pipettes and storage tubes were used. Aseptic precautions were taken to avoid contamination. As a 'fresh' control of activity, 0·2 c.c. of the mixed sample was taken immediately for measurement of respiration. The rest of the sample was divided among several small tubes (0·25 c.c. in each) and covered with a layer of liquid paraffin to prevent evaporation and rapid gaseous exchange with the air. These separate samples were then treated in various ways according to the experiment. After treatment respiration was measured.

The instrument used to measure oxygen consumption was the modified Barcroft respirometer devised by Dixon (1934). The bottles were of standard size (about 40 c.c. capacity). A scroll of filter paper soaked in 20 % KOH and placed in the inner cup served to absorb CO_2. The amount of oxygen consumed in a given time was read from the gas pipette attached to the respiration bottle. The respirometers were mounted on a shaker and immersed in a thermostatic bath kept at 37° C. The standard procedure adopted for both fresh control and treated semen was to put 0·2 c.c. of semen into a Barcroft bottle containing 3·8 c.c. of dilutor. The formula for this dilutor is given by Milovanov (1934), and is the best we have yet tried for the measurement of respiration. The composition is as follows:

Na_2HPO_4 (anhyd.)	0·824 g.
KH_2PO_4 ...	0·08
Glucose ...	3·20
Glass distilled water	100·0 c.c.

The oxygen consumption was recorded at 10 min. intervals for a period of 2 hr. After reduction to S.T.P. the oxygen consumed was expressed, for comparison with the fresh control, in terms of the volume of semen or, for an absolute measurement, in terms of the number of spermatozoa. The 'density', i.e. the number of spermatozoa per unit volume, was measured by the standard haemocytometer technique. The variation in number of spermatozoa in separate samples of 0·2 c.c. from the same original mixed collection, and measured after respiration, is within the experimental error of sampling. The total oxygen consumed in 2 hr. by a fresh sample varied from about 0·247 to 0·327 c.c. and, per million spermatozoa, from about 0·470 to 0·690 cu.mm. The rate of oxygen uptake of a fresh sample of semen is practically constant over 2 hr., but that of a sample, adversely affected, may decline after about half an hour. Thus in taking the total oxygen consumption in 2 hr. as a measure of the damage done to the spermatozoa, two factors are involved, the effect on the rate of oxygen consumed and the effect on the survival of the spermatozoa in the respirometer. Since there is some variation in samples collected at different times, each treated sample was expressed as a percentage of the fresh sample from the same mixed collection.

After respiration had been measured, a drop of the sperm suspension from the Barcroft bottle was examined under the microscope. The degree of motility and the proportion of spermatozoa exhibiting motility were recorded. Within the limits of these rough eye measurements there was close agreement between the motility of the sample and the oxygen consumption. For example, a sample which had consumed only 20 % oxygen as compared with a fresh sample showed a large proportion of the spermatozoa to be dead and only a small number of feebly active spermatozoa; while a drop from a fresh sample, or one from a sample which still showed high respiratory activity, showed nearly all the spermatozoa in rapid progressive motion.

1. TEMPERATURE SHOCK AND ACCLIMATIZATION

Semen was manipulated in the warm chamber at 30° C. 0·2 c.c. was added to the Barcroft bottles and cooled in different ways, then warmed, diluted and the respiration measured. In test I the semen was added to warm bottles in the chamber, and then these bottles stood for 10 min. in air chambers kept at controlled temperatures. In test II the warm bottles, containing the semen, were stood in water at the same controlled temperatures. In test III the semen was added to bottles previously cooled, and in test IV the semen was added to cooled dilutor. Table 1 shows the results of this experiment. The results clearly indicate that the spermatozoa are sensitive to a sudden drop in temperature, and that the lower the temperature to which the semen is cooled, the greater is the harm done. From this preliminary experiment we realized the justification for taking special care to avoid accidental cooling. For instance, test III demonstrated the danger of temperature shock to semen collected in the ordinary glass receiver during winter. The spermatozoa will suffer if ejaculated into a cold receiver even at a temperature of 15° C. A comparison of tests I and II demonstrates that rapid conduction of heat from a glass bottle will adversely affect the spermatozoa. Test IV illustrates the danger of adding spermatozoa to cold dilutor.

TABLE 1. EFFECT OF TEMPERATURE SHOCK ON RAM SPERMATOZOA

(Respiration as percentage of uncooled sample)

Temperature lowered to ° C	Test I Warm bottles placed in cold chambers	Test II Warm bottles placed in cold water	Test III Cold bottles	Test IV Cold dilutor
15	89	92	77	77
10	78	67	69	77
5	62	58	36	24
1	54	37	26	15

The results obtained in this preliminary experiment do not differentiate between three possible factors which may be involved in sudden cooling. These are (*a*) the harmful effect of low temperature itself, (*b*) the rate of cooling of the spermatozoa, and (*c*) the time taken by the spermatozoa to become acclimatized to a given temperature change. The procedure in a more complete experiment was as follows. Semen was manipulated in the warm chamber at 27° C. 0·2 c.c. was taken for immediate measurement of respiration, and the rest divided into portions (0·25 c.c. in each small test-tube) and covered with liquid paraffin. The heater in the chamber was then turned off and the temperature allowed to fall gradually to 20° C, which it did in about 25 min. After remaining at 20° C for 10 min., the samples were then placed successively in thermostatic chambers at 15, 10, 5 and 1° C. The time the samples remained in each chamber was varied according to the experiment. After reaching 1° C the samples remained in the chamber until next day (about 24 hr.) and were then stood at room temperature for 20 min. and finally warmed to 27° C for 10 min. in the warm chamber. The respiratory activity was then measured in the usual way. The results are set out in table 2 and the mean respiratory activities at various rates of cooling plotted on the accompanying graph (figure 1). It will be seen from the data and the curve that rapid cooling (less than 5 min. in each chamber) reduces the respiratory activity, measured after 24 hr. storage at 1° C, to about 20 % of the fresh sample, but if kept for more than 2 hr. in each chamber the sample loses little or none of its respiratory activity. The results show that storage at a temperature as low as 1° C has no harmful effect provided the cooling is done gradually. The results also indicate that the rate of cooling is not so important as the time taken by the spermatozoa to become acclimatized to a given temperature change. This follows from the fact that when transferring the semen from one chamber to another, there is a lag of about 10 min. before the temperature

TABLE 2. EFFECT OF RATE OF COOLING ON SPERMATOZOA

(After storage for 24 hr. at 1° C)

(Respiration as percentage of fresh sample)

Minutes samples kept at 15, 10, and 5° C		Mean
2	22, 21, 18, 24	22
5	31, 20, 21, 16, 26, 30, 28	25
10	35, 41, 45, 41, 36, 33, 38, 28	37
15	44, 49, 35, 41, 40, 46	42
20	47, 54, 59, 68, 66, 63, 59, 50, 52, 61, 55, 58	53
25	72, 66, 54, 68, 67	65
30	76, 60, 66, 70	68
40	76, 73, 70, 80	75
60	75, 92, 95, 88, 89, 86	88
120	100, 100, 91, 97, 96, 100, 96, 100, 100	98
180	100, 100	100

of the spermatozoa in the test-tube reaches equilibrium with the temperature of the chamber. The actual rate at which the spermatozoa cool is determined by this lag, and is the same throughout the experiment, except perhaps when the changes are very rapid (less than 10 min. in each chamber). There is, moreover, no discontinuity at this part of the curve. The conclusion may be drawn that it is not so much the rate of fall of temperature which is the significant factor in gradual cooling, as the time which the spermatozoa remain at each given temperature and become acclimatized.

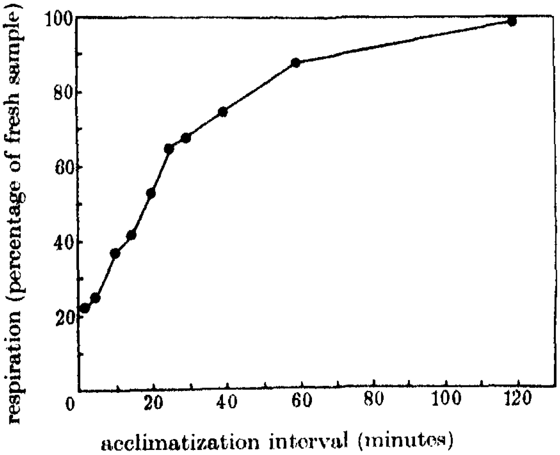

FIGURE 1. Effect of acclimatization interval upon the respiration of ram spermatozoa kept successively at 15, 10 and 5° C before storage for 24 hr. at 1° C.

TABLE 3. EFFECT OF SUDDENLY COOLING THROUGH 10° C DURING GRADUAL COOLING

(Semen stored 24 hr. at 1° C)

(Respiration as percentage of fresh sample)

Gradual cooling (interval 20 min.) except for sudden cooling from °C	Test I	Test II	Test III	Mean
25–15	75	59	76	70
20–10	50	57	74	60
15– 5	44	52	65	54
10– 1	42	43	66	50

A further experiment provides evidence that temperature shock is more severe at a low temperature than at a higher one. Table 3 shows the results of suddenly cooling the semen through 10° C, while the sample is otherwise undergoing gradual cooling. It will be seen that while sudden cooling is harmful at all temperatures, the effect is most marked at the lower ones.

For short exposures temperature shock is relatively less severe than for long exposures. This is shown in the following experiment in which the semen was cooled rapidly and stored for only 1 hr. The results are shown in table 4. At temperatures above 15° C there is little effect, while at a temperature as low as 1° C the respiratory activity still remains at about 45% of its original value. The effect of temperature shock is therefore accumulative and takes some time to reach its full effect.

TABLE 4. EFFECT OF TEMPERATURE SHOCK ON SPERMATOZOA STORED AT DIFFERENT TEMPERATURES FOR 1 HR.

(Respiration as percentage of fresh sample)

Sudden cooling to °C	Test I	Test II
20	100	100
15	98	100
10	85	81
5	61	78
1	42	53

Temperature shock is only apparent when the temperature is suddenly lowered; there is no corresponding effect when the temperature is suddenly raised. The results of some tests which justify this conclusion are shown in table 5. Sudden warming was done by taking the sample straight from 1° C to the water-bath at 37° C. Slight differences between the pairs, slowly and rapidly warmed, are not significant.

TABLE 5. EFFECT OF SUDDEN WARMING ON SPERMATOZOA

	Stored at 1° C for 5 hr.	Stored at 1° C for 24 hr.
Sudden cooling (no acclimatization):		
Sudden warming	47	20
Gradual warming	52	19
Gradual cooling (acclimatization interval 20 min.):		
Sudden warming	78	52, 58, 66
Gradual warming	76	54, 68, 63
Gradual cooling (acclimatization interval 120 min.):		
Sudden warming	—	96, 100
Gradual warming	—	100, 100

Discussion

The results obtained justify, so far, the following conclusions. A low temperature (1° C) is not harmful to spermatozoa provided the cooling is done gradually. Sudden cooling (temperature shock) has a deleterious effect. The effect takes some time to reach its maximum: a short exposure to a low temperature being less harmful than a long one. The effect increases as the temperature, at which sudden cooling occurs, is lowered. No harm is done to the spermatozoa if the temperature is lowered gradually by stages of 5° C, and there is a sufficiently long interval (2 hr.) between each stage. Acclimatization is therefore a slow process which takes some time to reach completion. There is either no temperature shock when spermatozoa are raised to a higher temperature or, in this case, acclimatization takes place practically instantaneously. In a sample of semen subjected to temperature shock there is a decided reduction in the number of spermatozoa which reactivate on rewarming and a decrease in the activity of the cells which do reactivate. No visible change in the structure of affected cells has been detected.

We have as yet no explanation to offer for these phenomena, even the terms 'temperature shock' and 'acclimatization' are used empirically to express end results rather than processes, although we can say that both take some time to reach completion.

A suspension of spermatozoa is not a stable homogeneous system. It is in the first place composed of a heterogeneous population of cells with various ages, activities and powers of resistance. Moreover, metabolic processes are continually changing the composition of the cells and of the medium in which they are suspended. There is evidence that anaerobic and aerobic glycolysis occurs in a suspension of active spermatozoa, with depletion of glucose or other source of energy. There is accumulation of lactic acid, uptake of oxygen and formation of carbon dioxide. Cells *in vitro* also undergo a process of senescence, and their resistance decreases. All these processes have most probably different temperature characteristics so that a change of temperature alters the whole balance of the reactions. A sudden change of temperature will have an effect on the end result which is different from the effect of a slow change. It is probably along the line of investigation of these processes, and of their temperature characteristics, that an explanation of temperature shock and acclimatization is to be found.

Although the analogy may be superficial it is possible that the phenomena of temperature shock and acclimatization observed with mammalian spermatozoa are similar to those described by Mellanby (1940) for amphibian tissues and insects.

2. THE OPTIMUM TEMPERATURE OF STORAGE

Reference was made above to the different value given by various authors to the optimum storage temperature for mammalian spermatozoa. Our results show that the optimum depends, not only upon the actual temperature, but also upon the rate of cooling and the time of exposure.

TABLE 6. EFFECT OF STORAGE FOR 24 HR. AT 5 AND 1° C WITH SUDDEN AND GRADUAL (20 MIN. INTERVALS) COOLING

(Respiration as percentage of fresh sample)

	Test I	Test II	Test III	Mean
Gradually cooled to 5° C	88	75	60	74
Suddenly cooled to 5° C	70	55	51	59
Gradually cooled to 1° C	59	61	47	56
Suddenly cooled to 1° C	23	28	18	23

TABLE 7. EFFECT OF TEMPERATURE ON THE STORAGE FOR 6 DAYS OF SLOWLY COOLED RAM SEMEN

Storage °C	Test I	Test II	Test III	Test IV	Test V	Test VI	Test VII	Mean
15	0	0	—	0	—	—	0	0
10	24	11	—	13	—	—	0	16
7	32	—	—	35	41	39	22	34
5	72	67	82	49	67	72	51	66
3	72	—	—	66	69	79	80	73
1	67	78	76	69	71	76	82	74

If cooling is rapid, and the time of exposure short, table 4 shows that 20–15° C is the optimum. If kept longer at these temperatures, however, survival is not more than about 24 hr., as is seen in table 7. If the time of exposure is 24 hr., table 6 shows that 5° C is a more favourable temperature than 1° C for both sudden cooling and moderately slow cooling (20 min. interval). With very gradual cooling in stages of 5° C and with an interval of 2 hr. between stages, the optimum temperature is 1° C or possibly below, but there is not much difference between storage at 3 or 1° C. This is shown in table 7 and figure 2, which record the effect on respiratory activity of semen cooled gradually and stored for 6 days at various temperatures. At 1° C the slowly cooled semen retained 74 % of its original activity for 6 days. This figure, however, does not represent the full possibility of this method of storage. We have found that storing the whole quantity of semen in one tube gives better results than storing in small quantities. From two large samples, which were of exceptionally good quality, we have obtained 87 % of activity after 13 days and 82 % after 14 days.

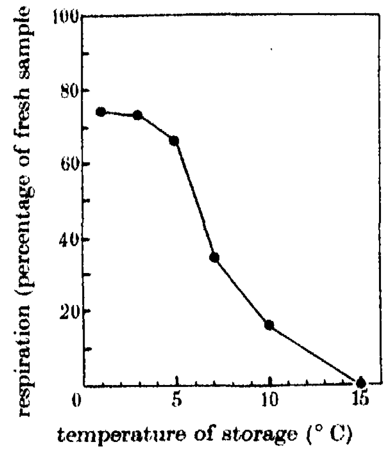

FIGURE 2. Effect of temperature of storage upon the respiratory activity of ram semen cooled gradually and stored for 6 days.

We wished to continue this study with long periods of storage, but with the advancing summer the quality of the ram semen declined, and further investigation has had to be postponed until the next breeding season in autumn. It will then be possible to test not only the survival of respiratory activity, but also, by means of artificial insemination, its fertilizing capacity.

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Molecular structure in relation to oestrogenic activity: derivatives of 4 : 4′-dihydroxydiphenylmethane

By N. R. Campbell

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The oestrogenic activity of four classes of 4 : 4′-dihydroxydiphenylmethane derivatives has been studied. Variation of activity in homologous series has been found, and maximum potencies established. The relationships between structure and maximum potency have been found parallel to those in other series of synthetic oestrogens.

The effect of methyl groups attached to the aromatic rings has been investigated, and the results suggest a biological connexion between these groups and other groups on the central carbon atom, since the effects vary in marked manner with alteration of the chain length of the central groups.

Dodds and Lawson (1938) noted that certain diphenylmethane derivatives, previously prepared by Easson, Harrison, McSwiney and Pyman (1934) for another purpose, possessed oestrogenic activity, when administered by injection in oil solution into ovariectomized female rats, in doses of 100 mg.

The oestrogenic activity of this class of compound has now been re-investigated, since earlier results appeared to suggest that activity depended essentially upon the presence of the 4:4′-dihydroxydiphenylmethane grouping, with but little modification by alkyl substituents on the central carbon atom. The activity is now shown to vary, in homologous series, as markedly as in the case of the important series of stilbene and diphenylethane derivatives described by Dodds, Golberg, Lawson and Robinson (1939).

The substances studied were prepared by direct condensation of an aldehyde or ketone with phenol or *o*-cresol, under the gentlest possible conditions, in the presence of hydrogen chloride.

Condensation of phenols with open chain ketones proceeded smoothly, several days being commonly required for a satisfactory yield, but crystallizations were generally easy to effect. The presence of a branched chain in the ketone had usually a depressing effect on the reaction, and greatly influenced the yield of diphenylmethane derivative. For example, di-*iso*-propyl and di-*iso*-butyl ketones under the standard conditions gave no product, although the normal ketones condensed satisfactorily. Similarly, although methyl-*iso*-butyl ketone condensed, methyl-*sec*-butyl and ethyl-*iso*-propyl ketones, in which the chain branching is α to the carbonyl, did not condense.

Cyclic ketones generally condensed easily, with the coproduction of troublesome, coloured, by-products. An α-methyl group greatly reduced the reactivity of the carbonyl group in these reactions.

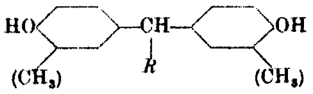
The significance of the results is best appreciated by studying the field of investigation in four sections. Four tables are given, each followed by discussion of the results.

Oestrogenic activities are given in terms of an arbitrary rat unit, which is taken as the minimum total weight of substance required to give 100 % full oestrus response in ovariectomized female rats when injected in six doses in sesame oil solution during 3 days.

It is necessary to emphasize that these figures are based on results from small numbers of rats—usually five—and, while giving a good basis for comparison under standardized conditions, must not be regarded as significant beyond the first digit.

The A series is derived from phenol and the B series from *o*-cresol.

1. PRODUCTS FROM ALIPHATIC ALDEHYDES:



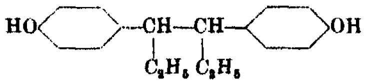
No.	Alkyl group *R*	Activity in rat units/g.	
1A	Hydrogen	20	Harden and Emmett Reid (1932)
2A	Methyl	20	Harden and Emmett Reid (1932)
3A	Ethyl	16	Harden and Emmett Reid (1932)
3B	Ethyl	8	Harden and Emmett Reid (1932)
4A	*n*-Propyl	40	Harden and Emmett Reid (1932)
5A	*iso*-Propyl	36	—
6A	*iso*-Butyl	100	—
6B	*iso*-Butyl	28	—
7A	Diethyl-methyl	140	—
8A	*n*-Hexyl	40	Harden and Emmett Reid (1932)
8B	*n*-Hexyl	4	Harden and Emmett Reid (1932)
9A	Di-*n*-propyl-methyl	90	—

These may all be regarded as α-alkyl-4:4′-dihydroxydiphenyl methanes, or systematically, as α:α-di-(4-hydroxyphenyl) alkanes. The former nomenclature facilitates rapid comparison of members of a series, but the latter is given in the experimental part.

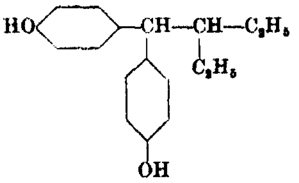
This section has not been studied so exhaustively as the second, since interest in it centred mainly on isomers of the much more active series of 4:4′-dihydroxydiphenyl ethanes or *vic*-di-(4-hydroxyphenyl) alkanes. Moreover, the activity found was less than in the aliphatic ketone condensation products studied in the second section.

The table shows that as the homologous series is ascended the oestrogenic activity remained essentially constant until $R = n$-propyl, when the activity was doubled. Branching of this chain had no effect (no. 5A), but addition of a methyl group in the β position to the carbonyl again doubled the activity (no. 6A).

With R = diethyl methyl (no. 7A) the activity again increased, reaching a maximum for the members of the series studied under this section. This substance produced an oestrus response in doses of 5–10 mg. in ovariectomized female rats and was considered particularly interesting, as a closely related isomer of $\gamma:\delta$-di-(4-hydroxyphenyl) hexane, commonly known as 'Hexoestrol':



$\gamma:\delta$-di(4-hydroxyphenyl) hexane
(hexoestrol)



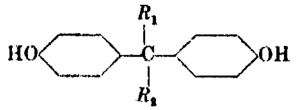
No. 7A

The relationship between these two substances is clearly such that the difference lies only in the transposition of an ethyl group and a 4-hydroxyphenyl group. This difference is sufficient to produce a fifty-thousandfold drop in oestrogenic activity, since hexoestrol, the most highly active oestrogen yet discovered, shows full potency in doses of 0·0002 mg. in ovariectomized female rats (Campbell, Dodds and Lawson 1939).

Increase in the length of R to the corresponding di-n-propyl methyl (no. 9A) slightly lowered the activity, but an intermediate, having $R = n$-hexyl, showed a distinctly decreased activity. Hence it would appear possible that the superior activity of no. 7A was due to its structural resemblance to hexoestrol.

The effect of nuclear methyl groups in the 3 and 3′ positions was interesting. The three cases studied were R = ethyl, n-hexyl and *iso*-butyl; the addition of the nuclear methyl groups considerably decreased activity. The first instance of this was noted by Dodds and Lawson (1938), but it will be shown that this effect is not generally found in other sections of the series.

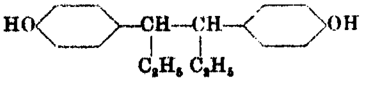
2. PRODUCTS FROM ALIPHATIC KETONES:

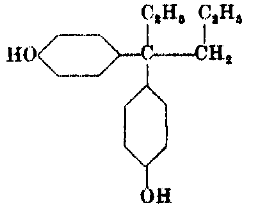


No.	Alkyl group R_1	Alkyl group R_2	Activity rat units/g.	
10A	Methyl	Methyl	36	Zincke (1905)
10B	Methyl	Methyl	40	Zincke (1913)
11A	Methyl	Ethyl	100	Easson, Harrison, McSwiney and Pyman (1934)
11B	Methyl	Ethyl	40	Easson, Harrison, McSwiney and Pyman (1934)
12A	Methyl	*n*-Propyl	200	Easson, Harrison, McSwiney and Pyman (1934)
12B	Methyl	*n*-Propyl	250	—
13A	Methyl	*n*-Butyl	40	—
13B	Methyl	*n*-Butyl	40	—
14A	Methyl	*iso*-Butyl	250	—
14B	Methyl	*iso*-Butyl	180	—
15A	Ethyl	Ethyl	200	Dianin (1891)
15B	Ethyl	Ethyl	1000	—
16A	Ethyl	*n*-Propyl	1000	—
16B	Ethyl	*n*-Propyl	4000	—
17A	*n*-Propyl	*n*-Propyl	500	Dianin (1891)
17B	*n*-Propyl	*n*-Propyl	5000	—
18A	*n*-Propyl	*n*-Butyl	200	—
18B	*n*-Propyl	*n*-Butyl	1000	—
19A	*n*-Butyl	*n*-Butyl	20	—
19B	*n*-Butyl	*n*-Butyl	200	—

These are conveniently regarded as $\alpha:\alpha$-dialkyl-4:4′-dihydroxydiphenyl methanes, and fall into two categories: the products from phenol (A) and those from *o*-cresol (B).

As R_1 and R_2 ascended their homologous series a maximum was found in both groups when R_1 = ethyl and R_2 = *n*-propyl, although in series B the maximum activity persisted in the next member ($R_1 = R_2 =$ *n*-propyl). The position of this maximum in A is of considerable interest, since this substance, no. 16A, is, once again, a closely related isomer of $\gamma:\delta$-di-(4-hydroxyphenyl) hexane:





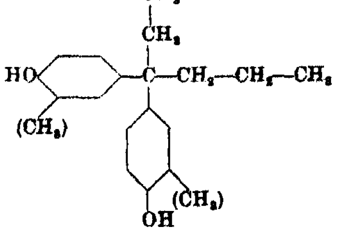
No. 16 A

The difference in this case lies in the transposition of a 4-hydroxyphenyl group and a hydrogen atom, and the drop in oestrogenic activity is approximately five-thousandfold.

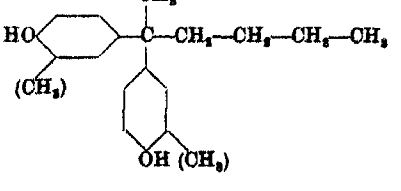
The significance of the position of the maximum in series B is less evident, since neither of the substances exhibiting maximum activity are isomeric with $\gamma:\delta$-di-(4-hydroxyphenyl) hexane. One differs from the most active member of series A only by the presence of two nuclear methyl groups (no. 16B) and the presence of two *n*-propyl groups in the other (no. 17B) recalls the 9:10-di-*n*-propyl-1:2:5:6-dibenzanthraquinol found by Cook, Dodds, Hewett and Lawson (1934) to be the most active of its homologous series. The maximum potency in B represents the highest oestrogenic activity yet found in the diphenylmethane series, and is about five hundred times as great as the maximum activity reported by Dodds and Lawson (1938) in their preliminary investigation of the series.

The effect of nuclear methyl groups in this section was in sharp contrast to the effect in section 1, since the most notable results indicated very marked increases. In one case only, no. 11 (R_1 = methyl, R_2 = ethyl), the activity was halved; in several cases the effect was small enough to be considered insignificant. The higher members of the series, however, were found to exhibit increases ranging from four and five-fold (nos. 15 and 16) to ten-fold (no. 19). These are the first cases noted where increase of oestrogenic activity occurs as a result of introduction of alkyl groups into a benzene ring in an active substance. Similar effects are discussed in the third section.

The very sudden drop in activity shown by no. 19 ($R_1 = R_2$ = *n*-butyl) appears to be attributable not merely to excessive overall chain length, but to the specific effect of the individual alkyl groups. This is suggested by the activity of no. 13 (R_1 = methyl, R_2 = *n*-butyl) being lower than that of its isomers no. 14 (R_2 = *iso*-butyl) and no. 16 (R_1 = ethyl, R_2 = *n*-propyl) or of its lower homologue no. 12 (R_1 = methyl, R_2 = *n*-propyl). It is also evident from this that the comparatively high activity of no. 16 is not merely a function of its overall chain length, but depends also upon the position of the phenolic substituents in the aliphatic chain:

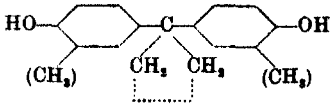


No. 16



No. 13

3. PRODUCTS FROM CYCLIC KETONES:



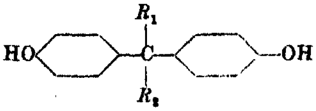
No.	Central cyclic group	Activity rat units/g.	
20A	*cyclo*-Hexane	40	Schmidlin and Levy (1910)
20B	*cyclo*-Hexane	40	Easson, Harrison, McSwiney and Pyman (1934)
21A	α-Methyl-*cyclo*-hexane	25	—
22A	*cyclo*-Pentane	15	—
22B	*cyclo*-Pentane	36	—
23A	α-Methyl-*cyclo*-pentane	100	—
24A	β-Methyl-*cyclo*-pentane	40	—

In this group only *cyclo*-hexane and *cyclo*-pentane rings have been included, and activities have not been found to approach those found in certain instances in the two previous groups. The product from *cyclo*-hexanone and phenol (no. 20A) was found more potent than that from *cyclo*-pentanone (no. 22A), but although a methyl group in the α position (no. 21A) decreased the activity of the former, a similarly placed methyl increased the activity of the latter approximately seven-fold (no. 23A). This increase was not so marked in the case of the β-methyl derivative (no. 24A), which showed a three-fold increase of activity.

Nuclear methyl groups in the 3 and 3′ positions in the *cyclo*-hexanone derivative (no. 20) had no effect, but produced slightly enhanced activity in the *cyclo*-pentanone derivative (no. 22).

It would appear that the presence of a methyl group in the molecule of a diphenylmethane of the *cyclo*-hexane type is not favourable to the production of oestrogenic activity, but that a methyl group in conjunction with a *cyclo*-pentane ring may have an enhancing effect. This applies whether the methyl group is attached to the benzene rings or to the *cyclo*-alkane group, but has most effect in the latter case when attached α to the aromatic substituents. In this connexion it is interesting to recall the structure of oestrone, in which the *cyclo*-pentane ring is associated with a methyl group which may be regarded as *angular* to one point of substitution and α to the other.

4. Condensation products containing additional aromatic rings:



No.	Group R_1	Group R_2	Activity rat units/g.	
25 A	Hydrogen	Phenyl	40	Russanow (1889)
26 A	Methyl	Phenyl	4	Zincke (1908)
26 B	Methyl	Phenyl	6	Easson, Harrison, McSwiney and Pyman (1934)
27 A	Ethyl	Phenyl	Nil	—
28 A	Phenyl	Phenyl	Nil	—
29 A	Hydrogen	Benzyl	100	—
30 A	Hydrogen	Diphenylmethyl	9	—

The addition of one benzene ring (no. 25 A) to the parent substance of the entire series (no. 1 A) produced a doubling of the activity (no. 25 A), but further addition, to form a tetraphenylmethane, completely removed activity (no. 28 A). A methyl group in addition to the third aromatic ring lowered the activity, while replacement of the methyl by ethyl once again removed activity completely.

Similarly, addition of one benzene ring to the substance 2 A (α:α-*di*-(4-hydroxyphenyl) ethane) increased activity considerably (no. 29 A), but a second aromatic ring (no. 30 A) lowered the activity again below that of the parent (no. 2 A).

No. 26 A was found to be little affected by methyl groups in the 3 and 3′ positions.

Experimental

General method I. The carbonyl compound (1 mol.) was mixed with phenol or *o*-cresol (4 mol.), treated with a few drops of concentrated hydrochloric acid (for 5–10 g. carbonyl compound), cooled in ice if necessary, and allowed to stand at room temperature. After a suitable period the mixture was freed from unchanged reactants by distillation in a current of steam, and the residue extracted with ether. The ether solution was dried over sodium sulphate, and the solvent removed. The residue, if not crystalline, was crystallized from benzene or benzene-light petroleum (b.p. 60–80°) and recrystallized several times from the same or other suitable solvent.

General method II. The mixture of carbonyl compound (1 mol.) and phenol or *o*-cresol (4 mol.) was cooled in ice and saturated with dry hydrogen chloride. The mixture was then allowed to stand and eventually worked up as under I.

Aldehydes and ketones employed were prepared by standard methods, or obtained from the usual sources.

Disubstituted acetaldehydes were prepared by the method of Stoermer (1906) and were not carefully separated from possible impurities, since these were not considered likely to affect the condensations.

Methyl-n-*butyl, di*-n-*butyl and* n-*propyl*-n-*butyl ketones* were prepared most conveniently from the corresponding carbinols by oxidation with potassium dichromate and dilute sulphuric acid.

Many straight chain aliphatic aldehydes have been condensed with phenols by Harden and Emmett Reid (1932).

Preparation of new substances

These are given their systematic names and may be referred back to the tables by means of the affixed characteristic numerals.

α:α-Di-(4-hydroxyphenyl)-β-methyl propane (no. 5A). *iso*-Butyraldehyde and phenol, method II—5 days, recrystallized from benzene (m.p. 152°). (Found: C, 79·5; H, 7·4. $C_{16}H_{18}O_2$ requires C, 79·3; H, 7·5 %.) Yield 30 %.

α:α-Di-(4-hydroxyphenyl)-γ-methyl butane (no. 6A). *iso*-Valeraldehyde and phenol, method II—10 days, recrystallized from benzene (m.p. 145°). (Found: C, 80·0; H, 7·95. $C_{17}H_{20}O_2$ requires C, 79·6; H, 7·9 %.) Yield 6 %.

α:α-Di-(3-methyl-4-hydroxyphenyl)-γ-methyl butane (no. 6B). *iso*-Valeraldehyde and *o*-cresol, method II—5 days, recrystallized from benzene-light petroleum (m.p. 124°). (Found: C, 80·2; H, 8·55. $C_{19}H_{24}O_2$ requires C, 80·2; H, 8·5 %.) Yield 3·5 %.

α:α-Di-(4-hydroxyphenyl)-β-ethyl butane (no. 7A). Crude diethylacetaldehyde and phenol, method II—5 days, recrystallized from benzene (m.p. 168°). (Found: C, 79·7; H, 8·25. $C_{18}H_{22}O_2$ requires C, 80·0; H, 8·2 %.)

α:α-Di-(4-hydroxyphenyl)-β-n-*propyl pentane* (no. 9A). Crude di-*n*-propyl acetaldehyde and phenol, method II—20 hr., recrystallized from benzene-light petroleum and from toluene (m.p. 128°). (Found: C, 80·5; H, 8·85. $C_{20}H_{26}O_2$ requires C, 80·5; H, 8·8 %.)

β:β-Di-(3-methyl-4-hydroxyphenyl)-pentane (no. 12B). Methyl-*n*-propyl ketone and *o*-cresol, method II—5 days, recrystallized from benzene-light petroleum (m.p. 128°). (Found: C, 80·5; H, 8·55. $C_{19}H_{24}O_2$ requires C, 80·2; H, 8·5 %.) Yield 30 %.

β:β-Di-(4-hydroxyphenyl)-hexane (no. 13A). Methyl-*n*-butyl ketone and phenol, method II—6 days, distilled at low pressure (b.p. 210° 0·5 mm.), crystallized from benzene-light petroleum in refrigerator but liquefied at room temperature. (Found: C, 80·0; H, 8·6. $C_{18}H_{22}O_2$ requires C, 80·0; H, 8·2 %.)

β:β-Di-(3-methyl-4-hydroxyphenyl)-hexane (no. 13 B). Methyl-*n*-butyl ketone and *o*-cresol, method II—6 days, recrystallized from benzene-light petroleum (m.p. 104–105°). (Found: C, 80·65, H, 9·2. $C_{20}H_{26}O_2$ requires C, 80·5; H, 8·8 %.) Yield 15 %.

β:β-Di-(4-hydroxyphenyl)-γ-methyl pentane (no. 14 A). Methyl-*iso*-butyl ketone and phenol, method II—14 days, recrystallized from benzene and from acetic acid (50 % by vol.) (m.p. 153°). (Found: C, 80·2; H, 8·4. $C_{18}H_{22}O_2$ requires C, 80·0; H, 8·2 %.) Yield 20 %.

β:β-Di-(3-methyl-4-hydroxyphenyl)-γ-methyl pentane (no. 14 B). Methyl-*iso*-butyl ketone and *o*-cresol, method II—10 days, recrystallized from benzene (m.p. 128°). (Found: C, 80·3; H, 8·5. $C_{20}H_{26}O_2$ requires C, 80·5; H, 8·8 %.) Yield 10 %.

γ:γ-Di-(3-methyl-4-hydroxyphenyl)-pentane (no. 15 B). Diethyl ketone and *o*-cresol, method II—10 days, recrystallized from benzene (m.p. 120°). (Found: C, 80·1; H, 8·6. $C_{19}H_{24}O_2$ requires C, 80·2; H, 8·5 %.) Yield 25 %.

γ:γ-Di-(4-hydroxyphenyl)-hexane (no. 16 A). Ethyl-*n*-propyl ketone and phenol, method II—9 days, recrystallized from benzene and from benzene-light petroleum (m.p. 155°). (Found: C, 79·9; H, 7·8. $C_{18}H_{22}O_2$ requires C, 80·0; H, 8·2 %.) Yield 10 %.

γ:γ-Di-(3-methyl-4-hydroxyphenyl)-hexane (no. 16 B). Ethyl-*n*-propyl ketone and *o*-cresol, method II—14 days, recrystallized from benzene and from benzene-light petroleum (m.p. 90°). (Found: C, 80·2; H, 9·2. $C_{20}H_{26}O_2$ requires C, 80·4; H, 8·8 %.) Yield 30 %.

δ:δ-Di-(3-methyl-4-hydroxyphenyl)-heptane (no. 17 B). Di-*n*-propyl ketone and *o*-cresol, method II—10 days, recrystallized from benzene (m.p. 173°). (Found: C, 80·5; H, 8·75. $C_{21}H_{28}O_2$ requires C, 80·7; H, 9·0 %.) Yield 17 %.

δ:δ-Di-(4-hydroxyphenyl)-octane (no. 18 A). *n*-Propyl-*n*-butyl ketone and phenol, method II—9 days, recrystallized from benzene-light petroleum (m.p. 150°). (Found: C, 80·45, H, 9·1. $C_{20}H_{26}O_2$ requires C, 80·4; H, 8·8 %.) Yield 3 %.

δ:δ-Di-(3-methyl-4-hydroxyphenyl)-octane (no. 18 B). *n*-Propyl-*n*-butyl ketone and *o*-cresol, method II—9 days, recrystallized from benzene-light petroleum (m.p. 140°). (Found: C, 81·1; H, 9·35. $C_{22}H_{30}O_2$ requires C, 80·8; H, 9·3 %.) Yield 3 %.

ε:ε-Di-(4-hydroxyphenyl)-nonane (no. 19 A). Di-*n*-butyl ketone and phenol, method II—12 days, recrystallized from benzene-light petroleum and from benzene (m.p. 165°). (Found: C, 80·3; H, 8·9. $C_{21}H_{28}O_2$ requires C, 80·7; H, 9·0 %.) Yield 8 %.

ε:*ε*-*Di*-(3-*methyl*-4-*hydroxyphenyl*)-*nonane* (no. 19B). Di-*n*-butyl ketone and *o*-cresol, method II—12 days, recrystallized from benzene-light petroleum (m.p. 128°). (Found: C, 81·4; H, 9·45. $C_{23}H_{32}O_2$ requires C, 81·1; H, 9·5 %.) Yield 5 %.

1:1-*Di*-(4-*hydroxyphenyl*)-2-*methyl*-cyclo-*hexane* (no. 21A). *α*-Methylcyclohexanone and phenol, method II—14 days, recrystallized from benzene, from acetic acid and from anisole (m.p. 235°). (Found: C, 80·6; H, 7·5. $C_{19}H_{22}O_2$ requires C, 80·8; H, 7·9 %.) Yield 7 %.

1:1-*Di*-(4-*hydroxyphenyl*)-cyclo-*pentane* (no. 22A). *cyclo*-Pentanone and phenol, method II—18 hr., recrystallized from dilute alcohol, benzene and dilute acetic acid (m.p. 157°). (Found: C, 79·9; H, 7·15. $C_{17}H_{18}O_2$ requires C, 80·25; H, 7·15 %.) Yield 25 %.

1:1-*Di*-(3-*methyl*-4-*hydroxyphenyl*)-cyclo-*pentane* (no. 22B). *cyclo*-Pentanone and *o*-cresol, method II—18 hr., recrystallized from benzene and from dilute acetic acid (m.p. 162°). (Found: C, 80·45; H, 7·8. $C_{19}H_{22}O_2$ requires C, 80·8; H, 7·85 %.) Yield 5 %.

1:1-*Di*-(4-*hydroxyphenyl*)-2-*methyl*-cyclo-*pentane* (no. 23A). *α*-Methyl-*cyclo*-pentanone and phenol, method II—4 days, recrystallized from benzene-light petroleum and from benzene (m.p. 161°). (Found: C, 79·7; H, 7·2. $C_{18}H_{20}O_2$ requires C, 81·1; H, 7·5 %.) Yield 0·5 %.

1:1-*Di*-(4-*hydroxyphenyl*)-3-*methyl*-cyclo-*pentane* (no. 24A). *β*-Methyl-*cyclo*-pentanone and phenol, method II—18 hr., recrystallized from benzene (m.p. 171°). (Found: C, 81·3; H, 7·4. $C_{18}H_{20}O_2$ requires C, 81·1; H, 7·5 %.) Yield 11 %.

α:*α*-*Di*-(4-*hydroxyphenyl*)-*α*-*phenyl*-*propane* (no. 27A). Propiophenone and phenol, method II—5 days, recrystallized from benzene and from toluene (m.p. 176°). (Found: C, 83·0; H, 6·55. $C_{21}H_{20}O_2$ requires C, 82·85; H, 6·65 %.) Yield 1 %.

α:*α*-*Di*-(4-*hydroxyphenyl*)-*β*-*phenyl*-*ethane* (no. 29A). Phenylacetaldehyde and phenol, method I—18 hr., recrystallized from benzene and from toluene (m.p. 140°). (Found: C, 83·1; H, 6·7. $C_{20}H_{18}O_2$ requires C, 82·7; H, 6·3 %.) Yield 10 %.

α:*α*-*Di*-(4-*hydroxyphenyl*)-*β*:*β*-*diphenyl*-*ethane* (no. 30A). Crude diphenylacetaldehyde and phenol, method II—18 hr., recrystallized from benzene and from toluene (m.p. 236°—decomp.). (Found: C, 85·5; H, 6·3. $C_{26}H_{22}O_2$ requires C, 85·2; H, 6·05 %.)

I wish to thank Professor E. C. Dodds for facilities for carrying out this

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END OF THE ONE HUNDRED AND TWENTY-NINTH VOLUME (SERIES B)

ABSTRACTS

OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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12 JUNE 1940

The wave-form of atmospherics at night. By J. S. ELDER, D. B. HODGES, W. E. PHILLIPS, B. F. J. SCHONLAND, F.R.S. and J. W. VAN WYK. (*Received* 5 *February* 1940.)

The wave-form for all atmospherics received at night from sources within 2000 km. can be accurately described as a ground-pulse followed by a series of sky-pulses produced by successive reflexions between the ionosphere and the earth, 30 such reflexions being frequently recorded. The time separation between the peaks of these pulses is determined by the distance travelled and the height of the layer. The primary pulse emitted by the source is usually a single complete oscillation of period ranging from 50 to 400 μsec. At distances greater than 500 km. the ground-pulse and the first sky-pulse merge owing to the shortness of the time-interval between them. Differences of amplitude, form and phase between pulses can arise from differences in angle of emission from the parent lightning channel.

The height of the reflecting layer can be determined within ± 1 km. It ranged from 85·5 to 90·5 km. during two winter months, with a mean of 88·0 km. The distances of the sources as found by analysis of the pulse series were corroborated by independent location with cathode-ray direction-finders. The reflexion-coefficient of the layer for the pulses of longer period exceeded 0·80. The velocity of the ground-pulse where it can be tested is within 0·7 % of that of light.

Operator calculus in the electron theory of metals. By K. Fuchs. (*Communicated by M. Born, F.R.S.—Received* 28 *March* 1940.)

An operator calculus is developed applicable to problems in the electron theory of metals. It differs from the common operator calculus of the quantum theory in the fact that the wave function is defined in a finite space (the atomic polyhedron) bounded by a finite surface. This leads to the introduction of surface operators.

The position operator x cannot be developed with respect to the proper functions of the Hamiltonian. Instead, an operator ξ is introduced, which is essentially the Fourier development of x.

Thus there are three fundamental types of operators: The differential operator p, the multiplication operator ξ, and the surface operators. It is shown that with the help of these a consistent calculus can be developed.

The reversible formation of starch from glucose-1-phosphate catalysed by potato phosphorylase. By C. S. Hanes. (*Communicated by F. F. Blackman, F.R.S.—Received* 29 *March* 1940.)

An enzyme (phosphorylase), which catalyses the reversible conversion of starch and inorganic phosphate to glucose-1-phosphate, has been found to occur in a number of higher plants. A method is described for obtaining from potato tubers highly active preparations of phosphorylase which are free from certain enzymes which catalyse alternative reactions.

The reversibility of the transformations: starch + inorganic phosphate ⇌ glucose-1-phosphate, is shown by the fact that the reaction proceeds in either direction until the ratio of inorganic orthophosphate/glucose-1-phosphate attains a value which is not significantly altered by wide variations in the concentrations of the reactants or the enzyme. The equilibrium value of this ratio is not detectably affected by alterations in the concentration of starch but varies considerably with alterations in the concentration of hydrogen ion. This is shown to be due to the effects upon the extents of dissociation of the inorganic and esterified phosphate, the di-valent ions alone determining the equilibrium; thus the ratio $(HPO_4)^{=}/(C_6H_{11}O_5.O.PO_3)^{=}$ remained approximately constant at a value of 2·2 when the pH value was varied from 5 to 7. When glucose-1-phosphate is added to the enzyme there is an initial lag in the liberation of phosphate and the formation of starch. This induction phase is abolished, and the reaction velocity greatly increased, by small additions of starch or maltose.

The preparation on a large scale of glucose-1-phosphate (in the form of the crystalline potassium salt) by allowing crude potato juice to act in starch is described.

Observations on the polysaccharide formed from pure glucose-1-phosphate under the action of purified phosphorylase indicate that it resembles closely the so-called amylo-amylose fraction of natural potato starch.

The mechanism of this reversible interconversion and its bearing on the problems of carbohydrate metabolism in the plant are considered.

The effect of field size and pattern on the change of visual sensitivity with time. By B. H. Crawford, M.Sc. (*Communicated by Sir John Parsons, F.R.S.—Received* 29 *March* 1940.)

Results are recorded showing the effect of size of a circular conditioning field on brightness threshold measurements made at the centre of the field, firstly, with the field steadily exposed and viewed, secondly, when the conditioning field has been cut off and the eye is returning to a state of dark adaptation. Conditioning and test fields are both illuminated by white light. No field size effect is detectable over the range 60° diameter down to 3° diameter. Below 3°, for a steady state of adaptation, various rather complex effects of interference between the conditioning fields are shown; these are explained in terms of the effect of distant parts of the conditioning field in raising the threshold and a perceptual interference between the patterns of conditioning and test fields. For changing states of adaptation the effect of field size cancels out—except in an isolated case—if the initial conditioning field brightnesses are adjusted to give the same steady value of threshold: then, after cutting off these various conditioning fields, the decrease of brightness threshold follows the same course. However the test patch of retina be brought to a given steady state of adaptation, as measured by the brightness threshold, it will recover its dark adaptation in the same way. These considerations apply both to foveal vision and to vision in the parafovea at 8° from the fovea.

The pressure of water waves upon a fixed obstacle. By T. H. Havelock, F.R.S. (*Received* 29 *March* 1940.)

The diffraction of plane water waves by a stationary obstacle with vertical sides is examined, in particular the variation of amplitude along the sides and the average steady pressure due to the wave motion. Results similar to those in other diffraction problems are obtained for an infinite plane and for cylinders of circular or parabolic section, and approximations are made for sections of ship form. The examination was made in view of possible applications in the problem of a ship advancing through a train of waves, and the results are discussed in relation to the average additional resistance in such circumstances. It appears that the mean pressure obtained on diffraction theory from the second order terms can only account, in general, for a small proportion of the observed effect; the motions of the ship, and in particular its oscillations, are essential factors in the problem.

Ionic regulation of *Carcinus maenas*. By D. A. Webb. (*Communicated by J. Gray, F.R.S.—Received* 1 *April* 1940.)

Analyses have been made, with respect to the principal inorganic constituents, of the blood and urine of *Carcinus*, when living in normal sea water, diluted sea water, and sea water modified by the addition of magnesium sulphate.

The composition of the blood of individuals living in normal sea water is as follows (the concentration of each ion being expressed as a percentage of the concentration that would be expected if the blood were in dialysis-equilibrium with the external medium): Na 110 %, K 118 %, Ca 108 %, Mg 34 %, Cl 104 %, SO_4 61 %.

This ionic regulation is the resultant of the following processes: active absorption by the gills of sodium, potassium, calcium and chloride at a rate greater than that at which they are lost by diffusion; differential excretion by the antennary gland, which tends to conserve potassium and eliminate magnesium and sulphate; inward diffusion across the gills of magnesium and sulphate in accordance with the concentration gradient.

In normal conditions there is active absorption of water. In dilute media, when osmoregulation begins, this is suspended, and possibly there is a fall in the passive permeability of the gills to water. In other respects osmoregulation is brought about by an intensification of the processes responsible for ionic regulation.

The permeability of the cuticle under physiological conditions is such that it does not affect the salt and water exchange of the animal, which is controlled by the branchial epithelium.

The structure of the gills of four species of Decapoda is described and correlated with their powers of osmotic and ionic regulation.

The anatomy of ineffective nodules and its influence on nitrogen fixation.

By H. K. CHEN and H. G. THORNTON. *(Communicated by Sir John Russell, F.R.S.—Received 4 April 1940.)*

The anatomy and cytology of nodules produced on clover, peas and soy beans by "effective" and "ineffective" strains of *Rhizobia* were investigated, with especial reference to the changes in volume of the active infected tissue during the life of the nodule. In clover the mean volume of this active bacterial tissue is about three times as great in "effective" as in "ineffective" nodules. This is due to an early arrest of growth in nodules produced by ineffective strains. In all nodules the active bacterial tissue eventually disintegrates, but in effective clover nodules it remains without disintegration for about six times as long as in ineffective nodules.

In an experiment to test the nitrogen fixation by clover inoculated with an effective and an ineffective strain, the difference between the strains in the amounts of nitrogen fixed could be accounted for by the differences in volume and in duration of the active bacterial tissue. In peas, nodules produced by an effective strain were nearly twice the length as those produced by an ineffective strain and their bacterial tissue remained without disintegration for about twice as long. In soy beans the mean volume of bacterial tissue was 4·75 times as great in effective as in ineffective nodules and the percentage of that volume composed of infected cells was twice as great. In ineffective soy bean nodules disintegration of the bacterial tissue began when the plant was four weeks old and was practically complete by the twelfth week, at which time no disintegration could be found in effective nodules. The difference in amount of nitrogen fixed by soy-bean plants bearing each type of nodule could be accounted for wholly by the factors mentioned above.

Thus in both clover and soy-bean nodules the volume and duration of the active infected tissue are the main, if not the only, factors determining differences in nitrogen fixation, amongst the strains tested.

The soft X-ray spectroscopy of solids. II. Emission-spectra from simple chemical compounds. By H. M. O'BRYAN and H. W. B. SKINNER. (*Communicated by A. M. Tyndall, F.R.S.—Received* 9 *April* 1940.)

K- and *L*-emission bands emitted in the soft X-ray region of the spectrum by atoms in a number of chemical compounds have been investigated. The substances for which such spectra are available through the present or previous work are: fluorides, chlorides, bromides, iodides, oxides, sulphides, boron nitride and other boron compounds; and carbides. In the cases of SiC, BN and a number of oxides, spectra from both the component atoms forming the compound have been obtained.

The results are interpreted, as those for metals and element insulators have been in a previous paper, to give the characteristics of the bands of levels which exist for the valence-electrons in the normal state of the substance. In the case of the halides, data on the *p*- and *s*-levels of the negative ions are given, and it is shown that, even in this case, the crystal structure leaves its mark on the form of the bands of levels. The 2*p* bands of oxygen in the oxides are more spread-out on an energy scale, thus showing that the interaction between electrons in neighbouring atoms is considerable. The spectra of the metal ions in oxides are very complex, and an attempt is made to disentangle the factors which lead to this complication. In doing so, it is hoped that we may have thrown some light on the difficult subject of the structure of semi-polar compounds in solid form.

The strains produced by precipitation in alloys. By F. R. N. NABARRO. (*Communicated by N. F. Mott, F.R.S.—Received* 15 *April* 1940.)

If the process of diffusion in metals results simply in the interchange of pairs of atoms, large strains must be set up when a new phase precipitates in an alloy. The strain energy involved is calculated for particles of precipitate of various shapes, and is found to be least if the precipitate forms thin plates. The shape of the particle actually formed is influenced by this energy, by the degree of supersaturation of the solid solution, and by surface effects; the calculated shape is shown to agree reasonably well with that observed experimentally.

The fine structure of phloem fibres in untreated and swollen jute. By B. C. KUNDU and R. D. PRESTON. (*Communicated by V. H. Blackman, F.R.S.—Received* 16 *April* 1940.)

Studies of the optical properties of jute suggest that the wall is composed of chains of cellulose forming a single, fairly steep spiral. During growth the wall spiral becomes steeper up to the onset of wall thickening.

This fibre provides a study complementary to that of hemp. The outer layers have the least resistance to swelling and the phenomenon of "ballooning" is therefore never observed. For similar reasons transverse cracks never develop during swelling.

The results fully confirm the conclusion that swollen material cannot safely be used in determining wall structure.

Plant pigments in the Galápagos Islands. By T. W. J. Taylor. (*Communicated by Sir Robert Robinson, F.R.S.—Received* 18 *April* 1940.)

The Galápagos Islands were visited by the Lack-Venables Expedition during the winter 1938–39, and analyses were made of the anthocyanin pigments contained in the characteristic and largely endemic plants of Indefatigable Island. The methods used for anthocyanin identification under camp conditions are described. No new pigments were found, but a high proportion of the plants were found to contain nitrogenous anthocyanins. This provides powerful support for the views already put forward as to the distribution of such pigments, although slight modification of these views is needed.

Isomorphous replacement and superlattice structures in the plagioclase felspars. By S. H. Chao and W. H. Taylor. (*Communicated by W. L. Bragg, F.R.S.—Received* 19 *April* 1940.)

From X-ray oscillation—and rotation—photographs of a limited number of plagioclase felspars it seems probable that the structure-type depends upon the chemical composition in the following way. From pure soda felspar to at least 22 % lime felspar there extends an isomorphous series with the *albite* structure, based on a unit cell with linear dimensions 8 × 13 × 7 A approximately and containing four molecules of $NaAlSi_3O_8$ or the equivalent when (CaAl) replaces (NaSi). There is probably a similar range of isomorphism, near pure lime felspar $CaAl_2Si_2O_8$, with the *anorthite* structure which has axes 8 × 13 × (2 × 7) A, but is of course very similar to the albite structure. The characteristic features of the photographs of the intermediate plagioclases can be explained, with the help of an optical model, in terms of a structure in which sheets with the albite structure and sheets with the anorthite structure alternate, but more experimental data are needed for the complete elucidation of the problem.

The base-catalysed prototropy of substituted acetones. By R. P. Bell and O. M. Lidwell. (*Communicated by C. N. Hinshelwood, F.R.S.—Received* 23 *April* 1940.)

Kinetic measurements have been made at 25° on the catalysed halogenation of the following ketones: acetone, acetonylacetone, monochloroacetone, monobromoacetone, *as*.dichloroacetone and acetylacetone.

In all cases the reactions are of zero order with respect to the halogen and exhibit general basic catalysis. For the first two ketones general acid catalysis is also detectable. For each ketone the catalytic constants k_B of four carboxylate anions are related to the dissociation constants K_A of the corresponding acids by an equation of the type $k_B = G(I/K_A)^\alpha$, where G and α are constants for a given ketone. The catalytic constants measured cover a range of about 10^8.

Examination of the results (together with those of Pedersen for the bromination of acetoacetic ester and acetoacetic acid) shows that the exponent α decreases steadily from 0·88 to 0·48 as the reactivity of the ketone increases.

The catalytic coefficients of the water molecule are given approximately by the equations valid for the carboxylate anions, but the hydroxyl ion is in each case a much less efficient catalyst than these equations predict.

A thermodynamical theory of the tensile strength of isotropic bodies. By F. Fürth. (*Communicated by M. Born, F.R.S.—Received* 24 *April* 1940.)

By thermodynamic considerations a new formula connecting the tensile strength of an isotropic body with its melting energy and Poisson's elastic constant is developed and found in good agreement with experiments.

General bi-harmonic analysis for a plate containing circular holes. By A. E. Green. (*Communicated by G. I. Taylor, F.R.S.—Received* 24 *April* 1940.)

A general solution is given for problems of generalized plane stress distributions in an infinite plate which contains circular holes of varying sizes in any positions, subject only to certain conditions of convergence of the solution. The method for extending the results to allow for the effect of one or two straight boundaries is indicated.

As a particular case of the general solution the problem of the stress distribution in an infinite plate under tension containing three holes in a row is discussed, and a few numerical results are given. These results are compared with experiments which were carried out by Mr P. L. Capper. The comparison is incomplete as the experiments were done for a finite plate and the influence of the edges of the plate on the numerical values of the stresses is considerable. Agreement, however, is found for the general character of some of the stresses.

Smoking and tobacco pipes in New Guinea. By A. C. Haddon, F.R.S. (*Received* 25 *April* 1940.)

The combustion of aromatic and alicyclic hydrocarbons. V. The products of combustion of benzene and its monoalkyl derivatives. By J. H. Burgoyne. (*Communicated by A. C. G. Egerton, Sec.R.S.—Received 25 April* 1940.)

The progressive formation of products in the combustion of benzene and its monoalkyl derivatives has been studied by analytical methods, and the characteristic features of the isothermal reactions at various temperatures have been established.

A cool-flame reaction of *n*-propylbenzene has also been investigated, and by comparison with corresponding isothermal combustions, it is concluded that the propagation of cool-flames is conditioned by the accumulation of a phenylalkyl hydroperoxide.

The results are interpreted in the light of the theory of the two-stage process, and a schematic mechanism for the main combustion reaction is outlined. This comprises degradation of the side-chain (if present) and rupture of the benzene nucleus, followed by rapid degradation of the higher aliphatic aldehyde thus formed, yielding finally formaldehyde and the ultimate combustion products CO_2, CO and H_2O.

The mechanism of the semicircular canal. A study of the responses of single-fibre preparations to angular accelerations and to rotation at constant speed. By O. Löwenstein and A. Sand. (*Communicated by S. W. Kemp, F.R.S.—Received* 3 *May* 1940.)

Single-fibre discharges have been recorded from the horizontal ampulla of the isolated labyrinth of *Raja*. Responses to different speeds of angular acceleration and to rotation at constant angular velocity are described. The existence of a spontaneous rhythm of discharge which is increased by ipselateral, and inhibited by contralateral rotation is confirmed.

During uniform angular *acceleration* the frequency of impulses increases or decreases gradually in a linear manner, and the rate of increase or decrease is proportional to the speed of acceleration. The threshold speed of acceleration is in the region of 3° per sec.^{2}. During prolonged rotation at constant speed the frequency of discharge, having attained a maximum or minimum co-incidentally with the initial acceleration, gradually returns to the spontaneous value over a period of about 20–30 sec. These results are interpreted in terms of the physical properties of the cupula as described by Steinhausen.

The spontaneous discharge, and its excitation and inhibition by ipsilateral and contralateral rotation provide a basis for the interpretation of labyrinthine tone, and of the reflex responses to rotation in both directions of unilaterally operated animals. The sensory activity of the semicircular canal, interpreted in terms of the physical properties of the cupula, provides a framework which is adequate to account for the time relations of nystagmus and after-nystagmus.

ABSTRACTS

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18 JULY 1940

Melting and crystal structure. By J. W. H. OLDHAM and A. R. UBBELOHDE. (*Communicated by Sir William Bragg, P.R.S.—Received* 27 *February* 1940.)

The role of various types of lattice flaws is discussed in their bearing on melting and crystal structure. Freezing point determinations on lattices of a single molecule indicate the presence of such flaws in the crystals of polymethylene compounds. Freezing point determinations on composite lattices of ketone + paraffin and ketone + ketone emphasize the importance of the rotation of the group for melting, and indicate that lattice holes are easily formed, without marked lowering of the freezing point. Ordered composite lattices are formed when the number of holes does not exceed 12 % of all the methylene groups.

The results emphasize the importance of co-operative effects in lattice flaws. In the equilibrium state of a crystal, such flaws might arise both from energy effects at 0° K, especially in metals, and from energy and entropy effects at higher temperatures. A picture of melting is suggested in which a network of co-operative flaws breaks up the crystal into a mosaic, or aggregate of crystalline micelles, and in which the solid-liquid transition is comparable with a gel-s[illegible]

Molecular structure and rubber-like elasticity. By C. W. BUNN. (*Communicated by Sir William Bragg, P.R.S.—Received* 16 *April* 1940.)

The crystal structures of β gutta-percha, rubber and polychloroprene have been completely determined by interpretation of X-ray diffraction photographs. The probable molecular structure of α gutta-percha molecules has also been deduced.

Knowledge of the geometry of these molecules, together with certain features of the crystal structures, lead to a promising theory of the origin of rubber-like properties.

An examination of all the well-established structures of aliphatic molecules has led to the suggestion of a new stereochemical principle which appears to play a large part in determining the bond positions for successions of linked carbon atoms, just as the tetrahedral principle determines the bond positions of any one carbon atom. The principle is also obeyed in molecules containing nitrogen and oxygen atoms as well as carbon. The principle may be used (and has already been so used) to deduce the probable configurations of aliphatic molecules, and is especially valuable for chain polymers.

Potential energy curves in proton transfer reactions. By R. P. BELL and O. M. LIDWELL. (*Communicated by C. N. Hinshelwood, F.R.S.—Received* 23 *April* 1940.)

The potential energy curves involved in proton transfer reactions are discussed, with special reference to the base-catalysed prototropy of ketones. The assumption of covalent binding throughout is inadequate to account in detail for the relations observed between basic strength and catalytic power. Approximate calculations from first principles show that the ionic state may well be of importance in this type of reaction, and the energy curves constructed on this basis give a much more satisfactory picture of the experimental facts.

The distribution of autelectronic emission from single crystal metal points. By M. BENJAMIN and R. O. JENKINS. (*Communicated by R. H. Fowler, F.R.S.—Received* 29 *April* 1940.)

The field emission patterns from metal points show an electron distribution which can be related to the crystal lattice of the metal.

Surface atoms are mobile at relatively low temperature (the actual temperature being dependent on the nature of the metal). Owing to this mobility, the point is able to assume a shape of minimum free energy determined by the surface forces. This shape is the normal equilibrium form at all temperatures in the absence of external forces. It may be a roughly spherical surface with slight flats or hollows.

If an external field is applied, the surface atoms can be made to pile up over various parts of the surface. The degree of surface modification is a function of the strength of the applied field and of the temperature. The surface then is roughly spherical with slight hollows or elevations about 25 atoms in height. The surface can be frozen into any modification by maintaining the field and reducing the point temperature so that mobility ceases.

The flash-over phenomenon in high vacuum is discussed, and an explanation offered.

The effect of small traces of gas is illustrated and it is shown that the gas film is removed at a relatively low temperature 620–670° K.

The poisoning of a palladium catalyst by carbon monoxide. By M. G. T. BURROWS and W. H. STOCKMAYER. (*Communicated by D. L. Chapman, F.R.S.—Received* 29 *April* 1940.)

The combination of hydrogen and oxygen on the surface of a palladium catalyst at low partial pressures at the temperature of the laboratory is completely inhibited by very small amounts of carbon monoxide. The poisoning effect is only temporary and, during the induction periods which are observed, the carbon monoxide is removed by the oxygen in the mixture of hydrogen and oxygen. After the induction period combination proceeds at the normal rate. The length of the inert periods observed increases with the amount of carbon monoxide initially added to the system, and the rate of removal of the carbon monoxide increases as its pressure decreases and becomes comparatively very great when the carbon monoxide present does not exceed the amount required to cover the surface of the metal.

The observations recorded can be explained by a development of the hypothesis of de la Rive that the catalysis of the reaction between hydrogen and oxygen and between carbon monoxide and oxygen involves the alternate oxidation of the metal and the reduction of the surface oxide by hydrogen or by carbon monoxide respectively.

The reduction of ferric oxalate by isolated chloroplasts. By R. HILL and R. SCARISBRICK. (*Communicated by Sir Frederick Hopkins, F.R.S.—Received* 30 *April* 1940.)

Two methods have been used to measure the activity of chloroplasts isolated from *Stellaria media*, (*a*) the measurement with haemoglobin of oxygen produced from ferric oxalate, (*b*) the measurement of the rate of reduction of methaemoglobin in the presence of atmospheric oxygen, the methaemoglobin itself being reduced by the ferrous iron produced. The chloroplasts after isolation show a progressive fall in activity, approaching zero in 3–6 hr. Four different strains of the plant were grown which showed differences in the stability of the chloroplasts after removal. The Q_{O_2} measured as rate of oxygen production calculated on the basis of dry weight of leaf is about 20. The Q_{O_2} measured as rate of methaemoglobin reduction generally appeared less as the reduction of methaemoglobin by ferrous iron is relatively slow. The reduction of methaemoglobin in presence of ferric potassium oxalate has been studied quantitatively from the point of view of iron, methaemoglobin, and chloroplast concentration. The effect of different light intensities on the reduction of ferric oxalate by chloroplasts is similar to the effect of varying light intensity on photosynthesis in whole plants and lies within the range of values found by different workers. This ferric oxalate reaction is inhibited by urethane. Phenyl urethane inhibits in much smaller concentrations than ethyl urethane. The effective concentrations of urethane are similar to those affecting photosynthesis.

New studies on active nitrogen. I. By Lord Rayleigh, F.R.S. (*Received 3 May* 1940.)

This paper examines quantitatively the behaviour of nitrogen gas emitting the after-glow, under varied conditions.

The integrated light emitted under the most favourable conditions has been measured as 3·18 candle-sec. per c.c. of nitrogen, the (rarefied) nitrogen being reckoned as at N.T.P. The number of quanta emitted is estimated as about $1{\cdot}3 \times 10^{-3}$ of the number of molecules present.

It is concluded, in agreement with Kneser, that the addition of unexcited nitrogen to glowing nitrogen increases the (instantaneous) emission. It was found that a fivefold increase of total pressure produced about a fivefold increase of brightness. These tests were carried out at very low luminous intensities, when the spontaneous decay was negligible during an experiment.

If the active gas contained in 1 l. of weakly glowing nitrogen is allowed to diffuse quickly into an additional 1 l. volume of nitrogen, so as to dilute it by half, the candle power per unit volume is reduced about 4·3-fold. This is a fair approximation with the value 4, which would be expected if the reaction were bimolecular as regards active nitrogen, and is in agreement with the conclusion which has generally been drawn from observation on the rate of decay of the luminosity in closed vessels.

The effect of compressing the glowing gas has been re-examined, using a solid piston moving in a cylinder. It is now found that with the improved arrangements the brightness varies as the inverse cube of the volume. This is the logical conclusion from, and confirmation of, the previous experiments in which the concentration of (1) the inert nitrogen, and (2) the active nitrogen, are separately varied.

A further test was to expand the glowing gas into a doubled volume which was carried out by letting it pass into a supplementary exhausted vessel. This was expected from the previous result to reduce the intensity 8 times, but in fact did not reduce it more than 7 times. The gas was therefore somewhat brighter after the expansion than had been expected. Most sources of error would have the opposite effect, and there is an outstanding discrepancy in this result.

The effect of temperature on the nitrogen after-glow is examined quantitatively, maintaining the cooler and hotter portions in pressure equilibrium. Cooling to liquid air temperature, for instance, increases the brightness some 80 times. Most, but not all, of this is due to the additional concentration. Assuming (in accordance with the compression experiment) that the brightness is as the cube of the concentration, and correcting the results to uniform concentration, it is found that over the range examined the brightness varies as $T^{-0{\cdot}64}$ when T is the absolute temperature.

The positive temperature effect on ordinary chemical reaction corresponds to something more like T^{+100}.

New studies on active nitrogen. II. By Lord Rayleigh, F.R.S. (*Received 3 May* 1940.)

It is shown that pieces of sheet gold, copper, silver, or platinum may be made red hot or even melted by exposing them to active nitrogen produced in a low pressure discharge. The nitrogen gives up its energy to the metal, which remains unacted on.

This phenomenon is applied to measuring the energy of active nitrogen, drawing a known quantity of gas through the activating discharge and then over the metal, and measuring the energy liberated on the latter.

The amount of energy collected from the gas was surprisingly large, and is difficult to reconcile with existing theories of the nature of active nitrogen. In some cases the energy was as high as 10 eV for every molecule of nitrogen that passed through the discharge. This quantity of energy can with difficulty be accounted for by dissociation, even if it occurred to the extent of 100 %. The energy radiated as after-glow under favourable conditions is only of the order of 10^{-3} of the energy collected by the metal.

Two-phase equilibrium in binary and ternary systems. II. The system methane-methylene. III. The system methane-ethane-ethylene.

By M. Guter, D. M. Newitt and M. Ruhemann. (*Communicated by A. C. G. Egerton, Sec.R.S.—Received* 6 *May* 1940.)

The liquid-vapour equilibrium of the system methane-ethylene has been determined at five temperatures between 0 and − 104° C and over a wide range of pressures. The results are shown on pressure-composition curves and by a three dimensional pressure-temperature-composition diagram. The equilibrium of the ternary system methane-ethane-ethylene has also been determined at 0, − 78 and − 104° C. The relation between the phase behaviour of the two binary systems methane-ethylene and methane-ethane and that of the ternary system is discussed and illustrated by a pressure-composition diagram covering the pressure range 10–35 atm. and the whole range of compositions.

Investigations on the vibration spectra of certain condensed gases at the temperature of liquid nitrogen.

By G. K. T. Conn, E. Lee, G. B. B. M. Sutherland and C. K. Wu. (*Communicated by R. G. W. Norrish, F.R.S.—Received* 10 *May* 1940.)

A description is given of experimental methods which have been developed for the investigation of infra-red and of Raman spectra at the temperature of liquid nitrogen. The infra-red spectrometer is essentially the Pfund-Barnes type but it has been found possible to dispense with the fore-prism and to separate the overlapping orders from the grating by means of filter shutters. The spectrometer is used as a monochromator so that radiation only enters the absorption cell after it has been through the diffraction train. The absorption cell has been designed so that it may be used for high as well as low temperatures. The apparatus for the Raman effect is a modification of that introduced by De Hemptinne, being especially suitable for the examination of small quantities of material.

The infra-red and Raman spectra of hydrochloric and of deuterochloric acids at liquid nitrogen temperatures. By E. LEE, G. B. B. M. SUTHERLAND and C. K. WU. *(Communicated by R. G. W. Norrish, F.R.S.—Received* 10 *May* 1940.)

The infra-red absorption spectrum of solid hydrochloric acid has been obtained at the temperature of liquid nitrogen (i.e. approximately twenty degrees below its transition point) in the region of the fundamental frequency. The results obtained agree in general with the earlier observations of Hettner and disagree with those of Shearin who reported discrete rotation lines. The doublet band observed by Hettner has been resolved into two separate bands with maxima at 2701 and 2744 cm.$^{-1}$ and indications of a third band were obtained. Some fine structure, very different from that reported by Shearin, was observed on each of the two bands, which were unsymmetrical in shape, being steeper on the low frequency side. The Raman spectrum was obtained at the same temperature and also showed a doublet structure in contrast to earlier observations at higher temperatures which had given only a single line. The maxima of the two Raman lines lay at 2709 and 2759 cm.$^{-1}$. The explanation of this doubling is to be sought in the manner in which the hydrochloric acid molecules associate in the solid state, since the alteration in the fundamental frequency from that found in the gaseous state shows that strong interaction must exist. The absorption due to the fundamental frequency of deuterochloric acid was observed under the same conditions. Here three maxima were observed at 1965, 1982 and 1992 cm.$^{-1}$ and indications of more structure. These results show that the association of HCl and DCl molecules in the crystal below the transition temperature can probably take place in several different ways. Such a conclusion is in agreement with the X-ray investigations which revealed a symmetry so low that the crystalline structure could not be determined.

The kinetics of mutarotation in solution. By J. C. KENDREW and E. A. MOELWYN-HUGHES. *(Communicated by R. G. W. Norrish, F.R.S.—Received* 14 *May* 1940.)

The kinetics of the mutarotation of representative reducing sugars from the pentose, hexose and disaccharide series have been investigated polarimetrically over wide temperature ranges in aqueous solution.

The dependence of the velocity coefficient, k, upon temperature, T, is fairly well reproduced by an equation of the form

$$\ln k = C + (J/R) \ln T - E/RT.$$

E, the energy of activation at the absolute zero, is found to be some 6000 cal. greater than the apparent value afforded by the Arrhenius equation at room temperature. J/R has a value of -10, which is identified as the number of oscillators contributing to the activation.

The constants C, J and E of this equation are discussed, with reference to various reactions, in terms of a theory of unimolecular reactions in solution.

Foraminifera from the Green Ammonite Beds, Lower Lias, of Dorset.

By W. A. Macfadyen. *(Communicated by W. D. Lang, F.R.S.—Received* 14 *May* 1940.)

This paper is a contribution to the long continued researches on the Dorset Lias by W. D. Lang, F.R.S., who collected the described material.

Some 700 mounted specimens from the Lower Lias (*davoei* zone), of the Dorset coast have been studied. They are ascribed to six families, twenty genera, and fifty-five species; of these, eleven genera and forty-five species belong to the family Lagenidae. One genus and species, *Carixia langi*, and one other species, *Lagena davoei*, are described as new, and two new names are proposed to replace invalid names.

There were studied for comparison certain of the few described English Lias faunas, some described French and German material, and a number of well preserved but undescribed faunas from various Lias horizons and different English localities. This has yielded provisional evidence of the zonal ranges of the Dorset species. Some forms of *Frondicularia* were found to provide useful horizon markers.

A tentative correlation is given of the zoning of the Lias by various authors of papers on Lias Foraminifera; there are notes on the horizons of some described Lias faunas, and a review of previous work on British Lias Foraminifera.

Study of Jurassic Foraminifera has been comparatively neglected for many years, though there is a recent German revival. Some lengthy synonymies are therefore necessary. Certain ill-known forms are discussed in detail; they were inadequately described and figured, so that they have hitherto been wrongly placed. It has thus been possible to rectify the systematic position or status of the genera *Involutina*, *Problematina*, and *Bullopora*. The appearance in the Lias of *Bolivina* and *Plectofrondicularia* is demonstrated, genera usually stated not to occur in rocks of age earlier than the Cretaceous.

Foraminifera thrived in some of the Lias clays in which their shells are well preserved. There was a rapid evolution of new types, particularly of the predominating Lagenidae. In this family there appears to be wide variation within some of the groups, where neither "species" nor even "genera" are sharply defined.

The bulk of the paper is taken up by the systematic description of the Foraminiferal fauna; all the recorded forms are figured.

Inner excited states of the proton and neutron.

By W. Heitler and S. T. Ma. *(Communicated by N. F. Mott, F.R.S.—Received* 14 *May* 1940.)

The present meson theory exhibits a number of serious difficulties if applied to the interaction of fast mesons with nuclear particles. In order to avoid these difficulties the following hypothesis is made: Charge and spin of a proton and neutron shall be capable of assuming higher quantum states. Thus particles shall exist with approximately protonic mass and with charges $+2e$, $-e$, etc., and also with spins $3/2$, $5/2$.... It is assumed that the lowest of these higher states have excitation energies which turn out to be of the order of magnitude of 20 MeV. The higher spin states are introduced in the way of a simple model, namely that of a rotator with half-integer angular momentum. The theory is essentially non-relativistic with respect to the heavy particle.

The theory leads to the following results:

(i) The cross-section for scattering of mesons by a heavy particle is smaller by an order of magnitude than according to the former theory (and in qualitative agreement with experiments) and does no longer increase with energy. Thus no multiple showers are to be expected in this theory.

(ii) The anomalous magnetic moments of the proton and neutron diverge only logarithmically and, if the theory is limited to energies smaller than the rest energy of the proton, are in reasonable agreement with the experimental values.

(iii) The influence of the higher states on the nuclear potentials results in a deepening of the potentials at small distances. Even without introducing neutral mesons there is strong attractive proton-proton potential and it is discussed whether the proton-proton scattering data can be explained without this hypothetical particle.

(iv) The deviation from the Coulomb law for a proton derived from the former meson theory is much smaller in the new theory, whereas for the photo-disintegration of the deuteron the characteristic meson effects are expected to be even more marked.

The possibilities for observing the new particles are discussed. It is pointed out that it would have been unlikely had the particles been discovered in any of the experiments which have been carried out up to the present. Yet, one track published by Anderson and Neddermeyer, which cannot be due to any of the known particles, is tentatively interpreted as due to a particle with charge 2*e* and protonic mass.

Succinic dehydrogenase-cytochrome system of cells. Intracellular respiratory system catalysing aerobic oxidation of succinic acid. By D. Keilin, F.R.S. and E. F. Hartree. (*Received* 17 *May* 1940.)

The succinic dehydrogenase-cytochrome system, even in the cell-free colloidal preparation, behaves like a true respiratory system of the cell, showing the high catalytic activity and being affected by all the inhibitors in the same way and to the same degree as the respiration of intact cells. The efficiency of this system depends not only on the integrity of all its components but also on that of the colloidal structure which supports them and assures their mutual accessibility. The paper deals with the properties of each of the components of the system and with the mechanism of their interactions.

Bose-Einstein statistics and degeneracy. By D. S. Kothari and B. N. Singh. (*Communicated by M. N. Saha, F.R.S.—Received* 17 *May* 1940.)

The thermodynamic properties of degenerate and non-degenerate Bose-Einstein gas in the completely non-relativistic and the completely relativistic cases are derived. The relativistic degenerate case of Bose-Einstein statistics corresponds to black-body radiation. The properties and the possibility of the existence of non-degenerate radiation are discussed.

A study of sensitized explosions. V. Some new experiments on the hydrogen-oxygen reaction sensitized by nitrogen peroxide. By F. S. Dainton and R. G. W. Norrish, F.R.S. (*Received* 21 *May* 1940.)

The effect of pressure and temperature of reactants, of inert gases, of vessel diameter and of surface condition on the induction periods and explosion boundary of $2H_2 + O_2$ mixtures containing nitrogen peroxide have been determined in the temperature range 250–410° C. The principal results may be summarized:

(*a*) Increase of pressure causes the separation of the upper and lower limiting concentration of sensitizer to increase from zero, to a maximum value at the inversion pressure. Above this pressure the upper limit decreases and the lower limit increases linearly with increase of pressure. The induction periods of H_2-O_2-NO_2 mixtures of constant NO_2 content decrease rapidly with increase of pressure, approaching a small value asymtotically.

(*b*) All foreign gases lengthen the induction periods and eventually quench the ignition of an explosive mixture of H_2-O_2-NO_2 of constant composition. The order of efficiency of the non-reactant gases in lengthening the induction periods is $CO_2 > N_2 > A = He$, whereas the order of efficiency in quenching ignition is

$$CO_2 > He > N_2 > A.$$

(*c*) The induction period at the upper limit always exceeds the induction period at the lower limit. The reverse is true of the rates of the slow reactions in the vicinity of the limits.

(*d*) At constant total pressure rise of temperature causes the upper limit (P_u) to be raised according to the equation

$$\log P_u = E/RT + \text{a constant}.$$

E is 19,500 cal. at a total pressure of 75 mm. and increases with pressure to 25,600 cal. at 472 mm.

(*e*) At constant pressure the upper limit decreases and the lower limit increases as the reciprocal of the square of the diameter.

A study of sensitized explosions. VI. Experimental observations on the hydrogen-oxygen reaction sensitized by nitrosyl chloride. By F. S. Dainton and R. G. W. Norrish, F.R.S. (*Received* 21 *May* 1940.)

Small amounts of nitrosyl chloride lower the ignition temperature of $2H_2 + O_2$ mixtures by over 200° C; the efficiency in this respect of this substance being slightly greater than nitrogen peroxide. At a given temperature the ignition is confined between a lower and an upper concentration of catalyst, outside which only slow reaction occurs. Both the slow reaction and the ignition are preceded by an induction period, the length of which passes from large values through a minimum to further large values as the catalyst concentration is increased, and which, in contrast to the H_2-O_2-NO_2 system, is unaffected by irradiation with light from a mercury vapour lamp.

The induction periods and limits depend on the pressure and temperature of reactants in a very similar way to the induction periods and limits of the H_2-O_2-NO_2 system described in V. The similarity of the two systems extends to the effect of non-reactant gases in quenching the ignition: the quenching pressures being of the same magnitude in both systems. A difference is, however, found in that these foreign gases shorten the induction period in this system whereas they lengthen it in the H_2-O_2-NO_2 system.

A study of sensitized explosions. VII. A chain-thermal theory of the reaction between hydrogen and oxygen sensitized by nitrogen peroxide or nitrosyl chloride. By F. S. Dainton and R. G. W. Norrish, F.R.S. (*Received* 21 *May* 1940.)

Both these reactions are chain processes for which the net branching factor ϕ is inversely proportional to the induction period. By applying this relation to the experimental results described in V and VI, it is shown that ignition only occurs when the net branching factor attains a value determined by the sum of two quantities, one proportional to the thermal capacity and the other proportional to the thermal conductivity. It is pointed out that this ignition condition cannot be accounted for by any isothermal theory of ignition, but that it may readily be deduced from a thermal theory in which explosion is visualized as occurring only when the initial reaction rate, in a favourable volume element, is large enough to ensure that a critical temperature T_c is reached in a critical time t_c.

Theories of reaction kinetics are developed from these two systems which give ϕ in terms of the experimental variables. The expressions so deduced lead to a dependence of the induction period on these variables identical with that found experimentally. The effects of vessel diameter, surface condition, temperature, and concentrations of sensitizer, reactants and foreign gases on the explosion are satisfactorily accounted for by substituting these expressions in the ignition condition of the chain thermal theory.

X-ray studies of the molecular structure of myosin. By W. T. Astbury, F.R.S., and S. Dickinson. (*Received* 23 *May* 1940.)

Starting from the similarity between the X-ray diffraction patterns of muscle and myosin and those of mammalian keratin fibres, a detailed X-ray and elastic study has been carried out of strips of myosin film prepared by drying the sol.

Methods are described of orienting the myosin chain-molecules: when oriented but unstretched they are found to give an α-photograph almost indistinguishable from that of unstretched keratin, and when stretched a β-photograph almost indistinguishable from that of stretched keratin. As with keratin, the long-range elasticity rests on a reversible intramolecular transformation, the fully extended, or β-, form of the molecule being again about twice as long as the folded, or α-, form.

The X-ray and elastic properties of myosin are found to resemble most closely, not those of natural keratin, but those of keratin that has suffered breakdown among the cross-linkages (including disulphide bridges) of the polypeptide grid. For example, by pressing moist myosin at ordinary temperatures selective orientation is brought about analogous to what is produced in keratin only at higher temperatures. Similarly, myosin supercontracts in hot water or cold dilute alkali without the preliminary stretching treatment required by keratin.

The supercontraction of myosin has been studied quantitatively and the accompanying structural changes followed by X-rays. The results show that the effect cannot be explained as due simply to disorientation of long thin units: it must involve a further folding of the polypeptide chain system.

The hypothesis is put forward that the contraction of muscle arises from supercontraction of its myosin component. It will be shown in a subsequent paper that X-ray photographs of muscle in various states, both living and dead, are entirely consistent with this view.

The fact that myosin and keratin are similar in both molecular configuration and elastic properties is discussed in the light of recent X-ray and chemical findings, and the X-ray interpretation is given of the denaturation of myosin.

Anomalous viscosity. By A. S. C. Lawrence and J. R. Robinson. (*Communicated by E. K. Rideal, F.R.S.—Received* 25 *May* 1940.)

Anomalous viscosity is examined from first principles. A viscometer is described for the simultaneous measurement of viscosity and streaming birefringence; the latter property is used to give direct evidence of the nature of the orientation associated with the measured value of the viscosity under the same flow conditions. A number of sols have been examined and the results confirm the analysis. Predicted temperature effects have been confirmed. The effects of particle size, shape and concentration are considered with special reference to the conditions needed to obtain results which can be interpreted. Several related phenomena also receive explanations.

The colours of the plumose anemone *Metridium senile* (L.). By D. L. Fox and C. F. A. Pantin, F.R.S. (*Received* 30 *May* 1940.)

The anemone *Metridium senile* occurs in a number of striking colour varieties. These are to be found side by side in nature. The colours are inherited at least during asexual reproduction and do not depend on special food. The colour varieties are founded on three systems of pigment, black granular melanin in the endoderm, brown diffuse melanin in the ectoderm and red to orange lipochrome in fat droplets in both endoderm and ectoderm. In any variety each system may be present or absent. If all are absent we have a white form, if one is present we get red, brown or grey forms, if two or more are present we get other varieties. In combinations each pigment retains the distribution it has in the corresponding simple form.

The black endodermal pigment is a true melanin. The brown ectodermal pigment is also a melanin but is less stable. Even the white variety can be shown to possess a complete tyrosinase system when the tissues are finely ground. It is perhaps comparable to a case of dominant albinism.

The red and orange colours are due to a variety of lipochromes. The pigment of the red variety is chiefly the acidic carotenoid described by Heilbron, Jackson and Jones. It is related to astacene and we have named it "metridene". Yellow-orange anemones of the red series may contain considerable quantities of xanthophylls in addition to metridene esters. The only pigment of the white form is contained in the gonads and appears to be astacene.

Many of the carotenoids are present in too small quantities to influence the appearance of the animal. The assortment of carotenoids present varies considerably from one lot of *Metridium* to another. There is evidence that the occurrence of a variety of xanthophylls and carotene is more common in varieties which develop melanin. But there is no obvious correlation between the presence of a particular assortment of carotenoids and a particular distribution of melanin pigments. Flavines are not present in any of the colour varieties.

Coloured purine compounds are not present. The presence of significant quantities of uric acid was demonstrated and of this purine alone. It is present in the mesenteric filaments, in the yellowish excreted mucus, and granules of uric acid form white bands in the endoderm of the tentacles. In this position they contribute their whiteness to the total colour effect. Haematins do not contribute to the colour. Those that are present are derived from the ordinary intracellular respiratory systems.

The colours of the varieties are not an adaptation to the external environment as in warning coloration, etc., nor is there evidence as yet that the colour as such is of direct physiological importance to the animal. The varieties appear to illustrate Poulton's category of "non-significant colours", in which colour is a by-product of biochemical processes utilized for other purposes.

β-ray spectra of light elements. By A. A. TOWNSEND. (*Communicated by J. D. Cockcroft, F.R.S.—Received* 30 *June* 1940.)

A magnetic spectrometer has been used to investigate the β-ray spectra of some elements of low atomic number. It is found that all the spectra follow the Fermi distribution law for a certain range from the high energy end-point; the range over which this is true is much greater if the maximum energy is large. The end-points found are: C^{11} 0·981 ± 0·005 MeV, N^{13} 1·218 ± 0·004 MeV, Mn^{56} 2·88 ± 0·01 and 1·035 ± 0·015 MeV, Cu^{64} (positrons) 0·649 ± 0·004 MeV, Cu^{64} (negatrons) 0·574 ± 0·004 MeV, Zn^{63} 2·320 ± 0·005 MeV.

X-ray electrons expelled from metals by silver $K\alpha_1$ radiations. By C. J. BIRKETT CLEWS and H. R. ROBINSON, F.R.S. (*Received* 3 *June* 1940.)

The energies of the X-ray electrons expelled from targets of gold, platinum, silver and copper by silver $K\alpha_1$ rays have been measured in the magnetic spectrograph. The results have been combined with those of earlier measurements, and they yield a value for Planck's constant of $6{\cdot}62_9 \times 10^{-27}$ erg sec.

ABSTRACTS
OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

9 AUGUST 1940

A spectroscopic investigation of hydrocarbon flames. Parts II and III.

By W. M. VAIDYA. (*Communicated by M. N. Saha, F.R.S.—Received 15 May* 1940.)

The flames of hydrocarbons burning in atomic oxygen have been re-examined in order to find out whether the ethylene-flame bands which occur extensively in the flames of carbon compounds burning in air also occur in such atomic flames. The results show that ethylene, acetylene and benzene which gave the ethylene-flame bands burning in air also yield them when burnt in atomic oxygen. The intensities are, however, different. Methane does not react with atomic oxygen. Methyl alcohol gives CH, HO and the cool-flame spectrum of ether, while formaldehyde shows only HO bands.

The ethylene-flame bands are absent from the flame of benzene burning in atomic hydrogen, which means that oxygen is essential for their production. An emitter like CH_2 is, therefore, not possible.

The results obtained in the study of the spectra of hydrocarbon flames and the flames of intermediate products, burning in air and atomic oxygen, are discussed. It is suggested that, in hydrocarbon flames, the C_2 bands may be produced through the decomposition of a higher complex of carbon and oxygen such as C_3O_2 and the ethylene-flame bands from the decomposition of an oxy-compound formed by the direct incorporation of an oxygen molecule in a hydrocarbon molecule.

The nature of the emitter of the ethylene-flame bands is discussed.

The general problem of the mechanism of hydrocarbon combustion is discussed in the light of the spectroscopic data. It is concluded that the chain mechanism in which oxygen atoms had been assumed as chain carriers does not seem very likely; the evidence appears to be more in favour of the peroxidation theory.

The surface as a limiting factor in the combustion of hydrocarbons.

By R. G. W. NORRISH, F.R.S. and J. D. REAGH. (*Received* 17 *May* 1940.)

With special reaction vessels, varying widely in diameter while maintaining approximately constant volume, and employing several refinements in technique, a study has been made of the effect of surface on the slow oxidation of several hydrocarbons, both saturated and unsaturated. All reactions were of the degenerate branching type, and were found to be principally homogeneous in character. When the diameter of the reaction vessel was sufficiently reduced, the reaction rate was observed to drop abruptly toward zero, while the corresponding induction period increased toward infinity. Data so obtained have demonstrated that in narrow vessels surface deactivation can predominate over other processes of deactivation, while in wider vessels surface and volume deactivation occur to a comparable extent over a considerable range of pressure. When the vessel diameter is decreased to a critical value the surface deactivation almost alone can suppress the factors leading to chain branching, and from the reactions investigated the existence of such a critical diameter appears to be a general property of hydrocarbon oxidations in conformity with the theory of degenerate branching.

'Traces' of frictional contact and experiments on adhesion.

By R. SCHNURMANN. (*Communicated by Sir Harold Hartley, F.R.S.—Received* 4 *June* 1940.)

'Traces' due to frictional contact in the atmosphere can be made visible on glass plates through physical development by the condensation *in vacuo* of a metallic vapour. Above a certain load traces were obtained with silver, copper, steel, lead and cadmium.

The essential difference between traces of sliding and traces of rolling was demonstrated through microscopic observation. Traces of sliding are resolved into wide bands of predominantly parallel lines, whereas traces of rolling consist of numerous isolated specks. The minimum load required for the production of a trace of rolling is larger than for producing a trace of sliding. A more sensitive indicator of traces of rolling was arrived at by predeveloping glass plates.

The production of a trace on a glass plate is accompanied by the corresponding generation of a trace on the metal surface. This latter trace can be observed microscopically on the originally polished surface of a metal slider. Intentional lubrication, such as flooding the glass plate with distilled water or application of stearic acid, accentuates the traces.

Metals cast on to glass plates exhibit strong adhesion, unless they are appreciably superheated before casting. The contact area appears milky and full of blowholes, whilst the islands between the blowholes show an etched structure.

Evidence is produced that water vapour acts as an etching agent for the adhering metal surfaces. The adsorption layer trapped at the areas of 'contact' between etched metal and glass is considered to be sufficiently thin to be adhesive. Oxygen etching leads to a different result, since in this case the metal cracks off the glass plate on solidification leaving a trace behind.

The theory that local melting can occur at surface asperities on frictional contact is critically examined. Traces due to sliding, rolling, or strong adhesion were found under conditions which illustrate the importance of very thin adsorption and oxide layers, which the author considers cannot be reconciled with the existence of "welded metallic bridges".

Experiments on the elementary mechanism of solid friction. By R. SCHNURMANN. (*Communicated by Sir Harold Hartley, F.R.S.—Received 4 June* 1940.)

If deformation of surface irregularities were the predominant mechanism of solid friction, the coefficient of friction should have a measurable temperature coefficient. Apparatus is described, embodying the principle of the inclined plane, on which experiments in high vacuum were carried out over a wide range of temperature to determine whether such a coefficient could be detected.

Rust and adsorbed layers were found greatly to influence the measurements and a technique of cleaning the friction elements by prolonged baking out was developed. Thereafter, experiments on cadmium in the range between + 150 and − 100° C clearly foreshadow the existence of a temperature coefficient of static friction, computed at $-7\cdot5\times10^{-4}$ per $+1^\circ$ C.

Two-phase equilibrium in binary and ternary systems. IV. The thermodynamic properties of propane. By J. H. BURGOYNE. (*Communicated by A. C. G. Egerton, Sec.R.S.—Received 4 June* 1940.)

Existing physical and thermal data relative to propane have been summarized and correlated, and some new experimental determinations of pressure-volume-temperature relationships for the liquid at low temperatures have been carried out to make good deficiencies in the literature. On the basis of the information thus obtained the entropy and enthalpy of propane have been calculated for conditions of temperature between − 80 and 200° C, and at pressures of from 0·1 to 200 atm. The results are tabulated and also presented graphically on a temperature base.

The estimation of small quantities of carbon dioxide in air by the absorption of infra-red radiation. By H. DINGLE and A. W. PRYCE. (*Communicated by V. H. Blackman, F.R.S.—Received 6 June* 1940.)

This paper describes an investigation of the possibility of measuring small amounts of carbon dioxide in air by the absorption of infra-red radiation. It is shown that the method is simple; trustworthy and accurate, and is very sensitive for small amounts of carbon dioxide, of the order of that present in ordinary air. The principle is applied in a form superior to any hitherto used, in that the whole of the transmitted *radiation is measured instead of merely the radiation at the maximum of a particular* absorption band. This makes a spectrometer unnecessary, eliminates the disturbing effect of variation of temperature, and allows much less sensitive detecting apparatus to be used. The only preliminary treatment necessary is the removal of water vapour from the air under examination.

The growth of nodule bacteria in the expressed juices from legume roots bearing effective and ineffective nodules.

By H. K. Chen, H. Nicol and H. G. Thornton. *(Communicated by Sir John Russell, F.R.S.—Received* 6 *June* 1940.)

Strains of pea and soy-bean nodule bacteria, differing in their effectiveness in benefiting the host legume, were grown in media containing the unheated root juices from uninoculated host plants and from host plants bearing effective and "ineffective" nodules, and their growth was measured. The growth of the different bacterial strains on root juice from uninoculated plants was not correlated with their effectiveness. The juice from roots with effective nodules produced significantly better growth of the bacteria than juice from roots with ineffective nodules in 27 comparisons out of 44, the differences in the remaining comparisons being insignificant. The juice from roots with effective nodules produced significantly better growth than the juice from uninoculated roots in 10 comparisons out of 25 and significantly poorer growth in 3 comparisons. The juice from roots with ineffective nodules produced significantly poorer growth than the juice from uninoculated plants in 11 comparisons out of 25, and better growth in only 1 comparison.

The production, as a result of infection, of soluble substances affecting growth of the bacteria, affords an explanation of those differences in nodule growth that determine the effectiveness or ineffectiveness of the different strains of bacteria as regards nitrogen fixation within the host plant.

The actions of eserine-like compounds upon frog's nerve-muscle preparations, and conditions in which a single shock can evoke an augmented muscular response.

By S. L. Cowan. *(Communicated by E. B. Verney, F.R.S.—Received* 15 *December* 1939.)

The muscular responses to one shock, or to two shocks separated by 3–10 msec., applied to the nerve of a frog's nerve-sartorius preparation, when immersed in Ringer's solution, containing glucose and buffered with bicarbonate, were reasonably constant for about 3 hr. The main response to two shocks was followed by a small after-tension, which, like Bremer's "neuromuscular contracture", was abolished by atropine in concentration too small to influence the main response, or by curarine in concentrations which somewhat reduced the main response.

Treating nerve-sartorius preparations from frogs which had been kept at 14–18° C for some days before use ('warm' frogs) with prostigmine, eserine, or the dimethylcarbamic ester of 8-hydroxymethylquinolinium methylsulphate usually produced no change in the main response to one or to two shocks, but augmented the after-tension in the response to two shocks, and caused an after-tension to follow the response to a single shock. On the other hand, treating preparations from frogs which had been kept at 0–5° C for more than 40 hr. before use ('cold' frogs) with any of the eserine-like compounds caused the response to one or to two shocks to become augmented, as with mammalian preparations, and to be followed by irregular twitchings which lasted for about a second. The after-twitchings were abolished by atropine in concentrations which slightly reduced the main response, or by curarine in concentrations which reduced the main response rather more.

Treating 'warm' preparations with Ringer's solution of half the usual calcium content, or with Ringer's solution containing any of the following agents (which are known to sensitize to stimuli of long duration), in small concentration, produced *no appreciable change in the responses: tetraethylammonium iodide, sodium citrate,* guanidine carbonate, or methylguanidine hydrochloride. If, however, preparations so treated were also treated with any of the eserine-like compounds, their responses then became like those of a 'cold' preparation which had been treated with an eserine-like compound.

The responses evoked by applying one shock, or two with an interval of 3–10 msec. between them, to the pelvic end of the frog's sartorius muscle, after sufficient curarine to render nerve stimulation ineffective, were uninfluenced by any of the eserine-like compounds, or any of the five agents mentioned in paragraph 4, provided that shocks of short duration were used; when condenser discharges of long duration were used, treatment of 'warm' preparations with any of the five agents, in the same concentrations as produced the changes described in paragraph 4, augmented the response.

Calcium-deficiency, in large or small degree, affected little the response to stimulation of muscle. But a moderate deficiency changed the responses to repetitive stimulation of nerve in such a way as might have been imitated by stimulating the nerve of the untreated preparation at a lower frequency. When the deficiency was more severe, on stimulating with 150 shocks per sec., no response was visible for the first 1–3 sec., the tension then rose to less than a quarter of its normal maximum, and afterwards fell slowly. Prostigmine in large concentrations produced little improvement, whereas calcium quickly produced a marked improvement.

The actions of eserine-like compounds upon frog's nerve-muscle preparations, and the blocking of neuromuscular conduction. By S. L. Cowan. (*Communicated by E. B. Verney, F.R.S.—Received* 15 *December* 1939.)

The actions of prostigmine, eserine, and the dimethylcarbamic ester of 8-hydroxymethylquinolinium methylsulphate upon the frog's isolated nerve-sartorius preparation have been examined by a method developed by Lucas. With Ringer-soaked preparations from frogs kept at 14–18° C for some days before use the minimum interval at which two shocks applied to the nerve could elicit a summated muscular response was about 20 % longer than the absolute refractory period of the nerve. Any of the above-mentioned compounds prolonged the minimum interval for a summated response, but caused the time at which an extra interpolated shock began to cut down the response to the final shock to become only a little later. Curarine or atropine reversed the prolongation of minimum interval.

By the same method, the actions of the same eserine-like compounds upon preparations which had been treated with Ringer's solution of half the usual calcium content were examined. Either before or after treatment, it was impossible to cut down the muscular response by interpolating an extra shock.

The action of prostigmine upon Ringer-soaked preparations was examined by a method developed by Adrian, involving determination of the rate of recovery of excitability in the motor nerve at the site of stimulation and in the remaining more peripheral part of the preparation. Prostigmine influenced little the recovery process at the site of stimulation, whereas it prolonged the slower and more peripheral recovery process. Curarine reversed the prolongation of the more peripheral recovery.

With Ringer-soaked preparations, during the block of neuromuscular transmission produced by rapid repetitive stimulation of the nerve, the response of the muscle to direct stimulation was reduced. If, however, neuromuscular transmission had been blocked by curarine, stimulation of the nerve did not reduce the response of the muscle to direct stimulation.

On the turbulence of a tidal current. By J. Proudman, F.R.S. (*Received 12 June* 1940.)

The paper contains a theoretical investigation of the dynamics of a current when turbulence is of significance. The depth of water is taken to be uniform, while the external body-forces, the mean velocity of the current, and the statistics of turbulence are taken to be uniform over all horizontal planes, though they may vary between the sea-surface and the sea-bottom. The statistics considered are those involved in the forces of internal friction, the rates of doing work and the energy relations. The rotation of the earth and slow variations in time are allowed for.

After deriving a number of formulae which do not appear to have been previously placed on record, though they involve no new principles, a number of particular types of disturbance are studied. They are:

isotropic turbulence,
periodic disturbance, neglecting viscosity,
periodic disturbance, allowing for viscosity.

Only the last of these is able to account for the phenomenon of internal friction. One particular example is worked out, allowing for viscosity, viz. that of a steady current parallel to a vertical plane and uniform between the sea-surface and bottom. In this the forces of internal friction are proportional to a hyperbolic function of the depth below the sea-surface. To obtain a case more like that of actual tidal currents we should require the mean current to be such a function of the depth as would make the forces of internal friction proportional to the depth itself. But the investigation of such a function would require more elaborate mathematical methods than those used in the paper.

The flight of the dipterous fly *Muscina stabulans* Fallén.

By F. S. J. Hollick. (*Communicated by J. Gray, F.R.S.—Received* 17 *June* 1940.)

Females of the dipterous fly *Muscina stabulans* Fallén were chosen for an investigation of flight.

A comparison was made between the flight-system with the insect held stationary and in normal free flight, based on the resultant of those forces which maintain or change its state of motion during flight. Values for this resultant were determined in thirty-two individuals performing regular wing movements when held stationary in 'still air'. In all except four of these, the resultant force generated was equal to or greater than the weight of the insect. On an average, the line of action of the resultant was inclined forwards and upwards at 48° to the body-axis of the insect. The line of action cut the body-axis between 1·75 mm. in front to 3·25 mm. behind the centre of gravity, according to the amplitude of the wing beat. In the majority of cases the point of intersection was behind the centre of gravity in a position incompatible with stable free flight. This last result was confirmed by observing the path travelled by insects performing these wing movements when suddenly liberated in 'still air'. It was found that if the insect is moved forwards at the speeds of normal flight, with the body-axis inclined as in free flight, it continues in the same state of motion on liberation.

The effect of exposing the insect, held stationary, to a stream of air was investigated with the aid of a wind-tunnel. It was found that in those cases where the resultant force passes behind the centre of gravity of the insect in 'still air', the resultant is displaced forwards and comes to pass close behind, through, or in front of the centre of gravity, depending on the velocity of the stream of air and the inclination of the body-axis of the insect. There are good grounds for believing that the living insect held stationary in an appropriate stream of air closely resembles in essentials the system in free flight and is therefore suitable for further study of flight. In analysing further the forward displacement of the line of action of the resultant, it was found that the effect of the stream of air on the body of the fly was negligible in this connexion, as compared with that upon the wings.

By recording photographically the amplitude of wing beat for ten individuals, it was shown that the amplitude decreased with increasing airspeed and inclination of the body-axis—an effect which might be expected to produce a backward displacement of the resultant, that is, in a direction opposite to that observed. A study of the path travelled by the wing relative to the insect revealed a factor that would tend to produce the observed forward displacement of the resultant. It was observed that when the insect is exposed to a stream of air the course of the downward beat is displaced forwards, so as to convert the elliptical track, characteristic of wing movements in 'still air', into the figure '8' path, commonly associated with insect flight. This change in the path travelled by the wings was found to be dependent upon the movement or position of the third antennal joint relative to the second, resulting from the action of the stream of air on the third joint with its arista. The characteristic flight attitude of the legs, and the continued maintenance of wing vibrations when air was blown upon the insect from in front, were also found to be dependent upon the integrity of this system.

The path travelled by the wing relative to the insect is also dependent on the interaction of the sensory inflow from the antennae and halteres, since after mutilation of the latter the wing-movements associated with stimulation of the former were interrupted.

In the appendices are described: (I) the method of mounting living insects for experiment; (II) the design, calibration and use of an aerodynamic balance suitable for determining the magnitude of the vertical and horizontal components of the 'resultant' and the position of its line of action relative to the centre of gravity of the insect; and (III) a small wind-tunnel, with plate glass top and bottom, for observing the behaviour of the insect when exposed to a stream of air, and for recording photographically the amplitude of wing beat.

The quenching of the resonance radiation of sodium. By R. G. W. NORRISH, F.R.S. and W. MACF. SMITH. (*Received* 17 *June* 1940.)

The effective cross-section for quenching of sodium resonance radiation by the saturated and unsaturated hydrocarbons, tertiary amines and several diatomic molecules has been measured. The results indicate that with regard to quenching ability the gases fall into two groups, one comprising the saturated hydrocarbons and the inert gases and the other the unsaturated hydrocarbons and the amines. The difference in behaviour is too pronounced to be explained in terms of the discrepancy between the amount of energy the sodium atom gives up and the quenching molecule can receive but may be attributed to the presence of unsaturation in the molecule. Within any one series the number of atoms in the molecule apart from the unsaturated centre seems to have little influence on the quenching ability and it has been concluded that the quenching ability may be regarded as proceeding from a centre of unsaturation. The results have been qualitatively considered in the light of Stearn and Eyring's theory of non-adiabatic reactions, and in the terminology of the theory of the intermediate complex we may say that the presence of unsaturation manifests itself in a relatively large transmission coefficient.

On the specific heat of the sodium chloride crystal. By E. W. KELLERMAN. (*Communicated by M. Born, F.R.S.—Received* 21 *June* 1940.)

Calculations of about 280 proper frequencies of the sodium chloride lattice have been made previously. These frequencies have been used to determine the specific heat of NaCl according to Born's theory. It is shown that this number of frequencies is sufficiently large to make the determination of the frequency distribution and, therefore, of the specific heat possible. Agreement with experiment is obtained. It is shown in agreement with Blackman's investigations that the deviations from Debye's theory as found experimentally are due to Debye's assumption of a ν^2 law for the frequency distribution instead of distribution following from the atomic theory.

ABSTRACTS

OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

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9 October 1940

Catalytic activity, crystal structure and adsorptive properties of evaporated metal films.

By O. Beeck, A. E. Smith and A. Wheeler. (*Communicated by J. W. McBain, F.R.S.—Received* 4 *April* 1940.)

Metal films of high and reproducible catalytic activity were obtained by condensation of their vapours on glass at any desired temperature. The catalytic activity was measured by the hydrogenation of ethylene. The crystal structure of these films was investigated by electron diffraction. By controlling the pressure of an inert gas (N_2, A, etc.) during evaporation of the metals unoriented and oriented films could be produced at will, and their catalytic activities were compared.

Completely oriented nickel films were obtained with an inert gas pressure of 1 mm., the (110) plane, the least dense of the planes, lying parallel to the backing and the two remaining axes showing random distribution. Iron films were oriented with their (111) plane parallel to the backing, the least dense plane again thus oriented. Low-pressure adsorption of hydrogen at room temperature and of carbon monoxide at liquid air temperature revealed that the oriented gas evaporated nickel films have twice the available surface per gram of randomly oriented high-vacuum films but ten times the activity. The oriented films have therefore fivefold the activity of unoriented films. Oriented films of an available surface equal to unoriented but of fivefold activity could also be obtained in high vacuum by evaporation on to oriented films previously produced by evaporation in an inert gas. The activity per unit weight of the films was constant, indicating ready accessibility to the interior of the film by the reacting gases.

Adsorption of hydrogen was found to be immeasurably fast in all cases. Adsorption isotherms on nickel films were obtained for C_2H_4, CO, N_2, H_2 and O_2; and the effect of catalyst poisoning by CO and O_2, as well as the effect of sintering, was studied.

Loss of activity, decrease of hydrogen adsorption, and amount of poison were found to be proportional.

The most extensive studies were made on nickel films, but films of iron, cobalt, palladium, platinum and copper were also investigated and, with the exception of copper, similar results were obtained. The enhanced activity of oriented films appears to be associated with the larger distances in the (110) plane of nickel or the (111) plane of iron.

On the mechanism of boundary lubrication. Part I. The action of long-chain polar compounds.

By O. Beeck, J. W. Givens and A. E. Smith. (*Communicated by J. W. McBain, F.R.S.—Received* 4 *April* 1940.)

The effect of long-chain polar compounds on the coefficient of kinetic friction under boundary conditions has been studied using the Boerlage four-ball friction apparatus in various modifications. With steel balls of the highest grade, coefficients of friction for a great number of lubricants were measured as a function of the relative velocity of the rubbing surfaces.

The structure of thin films of these lubricants rubbed on polished mild steel surfaces was investigated by electron diffraction. It was found that lubricants showing no or little surface orientation had a constant coefficient of friction of about 0·1 over the available velocity range from 0 to 1 cm./sec. With oils which showed high surface orientation, imparted by addition of long-chain polar compounds, a sudden decrease of the coefficient of the friction was observed at various velocities of the sliding surfaces, depending upon the compound used. Investigation of a great number of compounds gave a direct correlation of this effect with molecular orientation: those compounds causing the effect to occur at the lowest velocities were found to be most highly oriented with their carbon chains most nearly perpendicular to the surface. Since such a change of the coefficient of friction can only be explained by the wedging of oil under the surface (oil drag), the effect was termed the 'wedging effect' leading to a type of lubrication which may be called 'quasi-hydrodynamic'. By measuring the electrical resistance between the sliding surfaces it was found that the regions of sudden decrease of the coefficient of friction correspond to a change from metallic contact to extremely high resistance.

The investigation shows that long-chain polar compounds act primarily by inducing the wedging effect and not by giving a direct protection to the surface.

On the mechanism of boundary lubrication. Part II. Wear prevention by addition agents.

By O. Beeck, J. W. Givens and E. C. Williams. (*Communicated by J. W. McBain, F.R.S.—Received* 4 *April* 1940.)

If two metal surfaces slide over each other in the presence of a lubricant and under high load, high pressures and temperatures prevail at those isolated spots which actually carry the load, leading to wear and possibly to breakdown. The action of wear-preventing agents under these conditions has been studied in detail, and it has been found that such agents are effective through their chemical polishing action, by which the load becomes distributed over a larger surface and local pressures and temperatures are decreased. Especially effective are compounds containing phosphorus or other elements of group V of the periodic system. These have been found to form a metal phosphide or homologue on the surface which is able to alloy with the metal surface, lowering its melting-point markedly, and by this action aiding greatly in maintaining a polish.

The wear experiments were carried out with a highly sensitive and accurate method which uses metal-plated steel balls as its sliding elements. Under the experimental conditions additions of 1·5 % triphenyl phosphine or triphenyl arsine in white oil gave wear-prevention factors of 7·2 and 12·2 respectively (relative to pure white oil). A further addition of 1 % of a long-chain polar compound is able to double the wear-prevention factor obtained with the polishing agents and wear-prevention factors as high as 17·6 have been observed. The specifically physical action of the long-chain polar compounds is discussed in the preceding paper.

The flame spectrum of carbon monoxide. By A. G. GAYDON. (*Communicated by A. C. G. Egerton, Sec.R.S.—Received* 13 *June* 1940.)

The spectrum of the flame of carbon monoxide burning in air and in oxygen at reduced pressure has been photographed on plates of high contrast which display the band spectrum clearly above the continuous background. Greater detail has been obtained than has been recorded previously and now measurements are given.

The structure of the spectrum has been studied systematically. It is shown that the bands occur in pairs with a separation of about 60 cm.$^{-1}$, this separation probably being due to the rotational structure. Various wave-number differences are found to occur frequently, and many of the strong bands are arranged in arrays using intervals of 565 and 2065 cm.$^{-1}$.

The possible origin of the spectrum is discussed. The choice of emitter is limited to a polyatomic oxide of carbon, of which dioxide is the most likely. The spectrum of the sub-oxide C_3O_2 shows some resemblance to the flame bands, but this molecule is improbable as the emitter on other grounds. A peroxide CO_3 is also a possibility, but no evidence for the presence of this has been obtained from experiments on the slow combustion of carbon monoxide.

Carbon dioxide in gaseous or liquid form is transparent through the visible and quartz ultra-violet, and the flame bands are not obtained from CO_2 in discharge tubes. Comparison with the Schumann-Runge bands of oxygen shows that it is possible that the flame bands may form part of the absorption band system which is known to exist below 1700 A if there is a big change in shape or size of the molecule in the two electronic states.

The electronic energy levels of CO_2 are discussed. Since normal CO_2 is not built up from normal CO and oxygen, an electronic rearrangement of the CO_2 must occur after the combustion process. Mulliken has suggested that the molecule in the first excited electronic state, corresponding to absorption below 1700 A, may have a triangular form.

The frequencies obtained from the flame bands are compared with the infra-red frequencies of CO_2. The 565 interval may be identified with the transverse vibration ν_2, indicating that the excited electronic state is probably triangular in shape. The

2065 interval cannot, however, be identified with the asymmetric vibration ν_3 with any certainty.

If the excited electronic state of CO_2 is triangular then molecules formed during the combustion by transitions from this level to the ground state may be 'vibrationally activated'. This is probably the reason for many of the peculiarities of the combustion of carbon monoxide.

X-ray structure and elastic strains in copper. By S. L. Smith and W. A. Wood. (*Communicated by G. W. C. Kaye, F.R.S.—Received* 18 *June* 1940.)

An X-ray examination of copper has been undertaken whilst the metal has been actually under tensile stress, and the X-ray structure investigated at a systematic series of points on the load-extension curve and during unloading and reloading from selected points on the curve. It is shown that the permanent strain is associated with the breakdown of the grains into the crystallite formation and that this change is essentially irreversible. The elastic strain of the metal is accompanied by reversible changes in dimensions of the atomic lattice which take place without leaving in the lattice any permanent distortion, as shown by the observation that the X-ray diffraction rings, including rings of the diffuse type, contract and expand under the action of the applied stress without any change in the degree of diffusion. The lattice-changes are distinguished in this way from certain lattice strains or lattice distortions permanently imposed on the lattice as a result of deformation by cold-work. Quantitative measurements are made on the elastic lattice strains exhibited by the (400) and (331) planes in a direction perpendicular to the axis of the applied stress, and these are compared with the equivalent external elastic constants. These measurements show that marked difference in rate of strain may take place in neighbouring grains subjected to the same external stress, and on this difference is based an explanation of the extensive breakdown of the grains into components of widely varying orientations which characterizes the structure of a polycrystalline metal after deformation beyond the yield point.

Infra-red absorption spectra of some amino compounds. By L. Kellner. (*Communicated by W. T. Astbury, F.R.S.—Received* 25 *June* 1940.)

An investigation of the infra-red absorption spectra of five amino compounds (glycine, diketopiperazine, tetramethyldiketopiperazine, glycylglycine and urea) has been made in the region $2{\cdot}8$–$3{\cdot}6\mu$. The substances were used in the form of thin crystalline layers deposited on quartz windows.

The spectra are discussed with regard to the molecular structure of the compounds under consideration. It is shown that the number and position of the N—H frequencies in glycine and glycylglycine confirms the view that these two molecules are in the zwitterion form in the crystal. The close similarity between the spectra of diketopiperazine and tetramethyldiketopiperazine on the one hand and the amino acids and urea on the other hand, proves that no lactamlactim interchange occurs in diketopiperazine and its derivative. Both compounds are shown to possess a centre of symmetry. It follows from the experimental evidence that in all investigated substances resonance between the C—N and C=O bonds takes place.

The vibrations and the molecular structure of urea and guanidonium.

By L. KELLNER. (*Communicated by W. T. Astbury, F.R.S.—Received* 25 *June* 1940.)

The vibrations of urea and guanidonium have been calculated for a field containing valence and angle forces. The assumption is made that urea has the symmetry C_{2v} and guanidonium C_{3h}. It is shown that it is possible to assign every observed frequency of these two substances to definite modes of vibrations under these assumptions. The force constants have been evaluated and have been found to be $f_{C-N} = 7\cdot1 \times 10^5$ dynes/cm. for guanidonium, and $f_{C-N} = 6\cdot6 \times 10^5$ dynes/cm. and $f_{C=O} = 9\cdot7 \times 10^5$ dynes/cm. for urea. These values are compatible with the hypothesis that quantum mechanical resonance occurs in both molecules, with the result that the C—N bond in urea has approximately 28 % double bond character and the C=O linkage a corresponding single bond character. The guanidonium ion shows complete resonance; each C—N bond has $\frac{1}{3}$ double bond character. Curves have been drawn to illustrate the relation between the valence force constants and the bond character.

Liquid jets of annular cross-section.

By A. M. BINNIE and H. B. SQUIRE. (*Communicated by R. V. Southwell, F.R.S.—Received* 25 *June* 1940.)

Experiments were made on a jet of water issuing from an annular orifice with a low velocity. The effect of surface tension was to cause the jet to coalesce after a short distance. The length of the annular portion was measured, and (when certain precautions were taken) was found to be in fair agreement with the predictions of an approximate theory.

The electrical polarization of frog skin by direct currents.

By O. GATTY. (*Communicated by E. K. Rideal, F.R.S.—Received* 27 *June* 1940.)

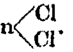
Data concerning the electrical polarization of frog skin by direct currents are presented with adequate statistical control. The results are discussed and show that the skin is electrically non-homogeneous and in a state of disequilibrium. The results are presumptive evidence for at least a three-unit model consisting of a series resistance and local action between an anodic area and a cathodic area as is also considered in the theory of corroding metals.

On stereochemical types and valency groups (Bakerian Lecture).

By N. V. SIDGWICK, F.R.S. and H. M. POWELL. (*Received* 27 *June* 1940.)

Theory and experiment agree that the arrangements in space of the covalencies of a polyvalent atom, while they are subject, from a variety of causes, to small variations seldom exceeding 5–10°, tend to conform to a quite limited number of types. Recent developments of mathematical physics have made it possible to calculate how these types are related to the electronic groups occupied by the valency electrons.

It is, however, not always possible to say which these sub-groups are, and it is in any case desirable for the chemist that he should be able to relate the spatial grouping to some more familiar property of the atom. The property here used is the size (in G. N. Lewis's sense) of the valency group of the central atom, and the number of shared electrons which it contains, together with that of the preceding (unshared) electronic group in the atom. The experimental results show the following relations.

I. When the valency group is less than 8 we have with a covalency of 2 a linear structure (as in Cl-Hg-Cl): with a covalency of 3 a plane with equal angles of 120° (BF_3): with 6 electrons of which only 4 are shared, as in the vapour form of stannous chloride, probably a triangular molecule  $\mathrm{Sn}\langle^{\mathrm{Cl}}_{\mathrm{Cl}}$.

II. With a complete octet the arrangement can be either tetrahedral or planar. When the covalency is less than 4 (partially shared octet) it is always derived from the tetrahedron, as in the triangular OH_2 and the pyramidal NH_3. The fully shared 4-covalent octet can be of either form. When the preceding electronic group, n in the grouping (n) 8, is 2, 8, or 18, it is always tetrahedral. In the transitional elements where $8 < n < 18$ it is found that if n is not much more than 8 the structure is tetrahedral, and if it is not much less than 18, planar; but the two series overlap, and for values of n of 13 to 15 and perhaps 16, both forms occur.

III. When the valency group is 10 with at least 2 of them (the 'inert pair') unshared, the structure of a dicovalent atom (as in $M[I_3]$) is linear; that of the 3-covalent (as in $C_6H_5.ICl_2$) is unknown; while that of the 4-covalent decet is found in the thallous and plumbous compounds to be planar, but in tellurium tetrachloride may possibly be a distorted tetrahedron.

IV. The peculiar 4-covalent duodecet in $M[ICl_4]$ is planar.

V. Covalency 5. So far as it has been examined (4 different types) this has always been found to give a trigonal bipyramid.

VI. Covalency 6. Three structures are theoretically possible: a trigonal prism, a trigonal antiprism, and a regular octahedron. Experimentally the structure is always found to be the octahedron, except in a few giant molecules such as those with the nickel arsenide lattice. The regular octahedron has been found with practically every possible size of the preceding group, as well as with the inert pair of electrons.

VII. A covalency of 7 has been examined in three or four compounds, which have two different structures, one derived from an octahedron and the other from the trigonal prism.

VIII. Covalency 8. The only compound examined, $K_4[Mo(CN)_8]$, is found (rather unexpectedly) to have a dodecahedral arrangement of the 8 CN groups.

Nearly (but not quite) all the structures can be even more simply related to the size of the valency group by assuming that the mean positions of the electron pairs in this group are the same whether they are shared or not, the structure being linear for 4 electrons, plane symmetrical for 6, either tetrahedral or plane for 8, a trigonal bipyramid for 10, and an octahedron for 12.

On maintained convective motion in a fluid heated from below.

By A. Pellew and R. V. Southwell, F.R.S. (*Received* 1 *July* 1940.)

This paper examines the stability in viscous liquid of a steady regime in which the temperature decreases with a uniform gradient between a lower horizontal surface which is heated and an upper horizontal surface which is cooled. The problem has been treated both experimentally and theoretically by Bénard, Brunt, Jeffreys, Low and Rayleigh, and it is known that instability will occur at some critical value of $gh^3\Delta\rho/\rho k\nu$, h denoting the thickness of the fluid layer, $\Delta\rho/\rho$ the fractional excess of density in the fluid at the top as compared with the fluid at the bottom surface, k the diffusivity and ν the kinematic viscosity. The critical value depends upon the conditions at the top and bottom surfaces, which may be either 'free' or constrained by rigid conducting surfaces.

The theoretical problem is solved under three distinct boundary conditions, and greater generality than heretofore is maintained in regard to the 'cell pattern' which occurs in plan. In addition, an approximate method is described and illustrated, depending on a stationary property akin to that of which Lord Rayleigh made wide application in Vibration Theory.

Mutation effects of ultra-violet light in *Drosophila*. By K. MACKENZIE and H. J. MULLER. (*Communicated by J. B. S. Haldane, F.R.S.—Received 8 July* 1940.)

Experiments are described which show that the frequency of sex-linked lethal mutations induced in spermatozoa of *Drosophila* by ultra-violet irradiation may be raised to approximately 5%. Among the important factors for obtaining such a result are the use of flies with a minimum of pigmentation, exposure through the ventral surface and the use for breeding of those individuals in which, as shown by their earlier death, the radiation has penetrated more effectively. Working by these methods, it has been found possible, by the use of a combination of filters, to determine that the region in which the mutational effect of ultra-violet light begins is between λ365 mμ and λ300 mμ, as expected for nucleic acid absorption. Further evidence has been adduced, by the use of an improved genetic technique, substantiating the earlier conclusion of the authors that these rays, although producing gene mutations with the frequency stated, fail to produce gross structural changes (rearrangements) of chromosomes in appreciable quantities. The genetic effect of ultra-violet radiation thus differs decidedly from that of doses of X and higher energy radiation of the same gene-mutational strength. Preliminary data also indicate that minute structural changes (rearrangements) of chromosomes resemble the gross structural changes and differ from gene mutations in being produced with difficulty, if at all, by ultra-violet light, as compared with X-rays. Cases of viable 'terminal deficiencies' also were absent. It seems very probable, therefore, that ultra-violet light cannot, or can only with difficulty, cause 'thoroughgoing' breakage of the chromonema, and that such breakage must occur in the production both of gross and of minute rearrangements but not in the process of gene mutation proper. If the latter process involves breakages at all, these would be of a different kind. It is argued that this evidence of a distinction between gene mutations and structural changes tends to favour the view that the 'genes' correspond to actual segments of the chromonema and are not mere conceptual isolates.

The influence of the solvent on the formation of micelles in colloidal electrolytes. Part I. Electrical conductivities of sodium dodecyl sulphate in ethyl alcohol-water mixtures. By A. F. H. WARD. (*Communicated by E. K. Rideal, F.R.S.—Received* 10 *July* 1940.)

In very dilute solutions in water sodium dodecyl sulphate behaves as a completely dissociated electrolyte. Above a concentration $c = 0{\cdot}00722$ N the equivalent conductance (Λ) falls sharply and micelles are formed. To throw light on the mechanism of micelle formation, the variation of Λ with c has been measured in a series of mixtures of water and ethyl alcohol. The curves for Λ against $\sqrt{c}$ show a gradual transition from the type associated with micelle formation to a uniform curve characteristic of strong electrolytes.

Addition of alcohol decreases the tendency to form micelles. When there is 40 % or more by weight of alcohol, micelles are no longer formed. The critical concentration first falls on addition of alcohol and then rises again. The effects of alcohol addition can be interpreted satisfactorily by considering the radius of the micelle to be equal to the length of the paraffin chain and thus to be independent of the concentration and nature of the solvent.

The energy changes involved in micelle formation are calculated. Aggregation can occur if the interfacial energy available from the destruction of the paraffin-solvent interfaces of the ions is greater than the work to be done against electrical repulsion. The interfacial energy is no longer the greater beyond 40 % of alcohol. It is shown that alcohol molecules are not in solution in the paraffin interior of the micelles in the mixed solvents, but are strongly adsorbed on the micelle surfaces.

The influence of regions of lowered dielectric constant around the micelles is considered. This is more important at high concentrations. The final fall in Λ with highly supersaturated solutions is attributed to the increased viscosity of the solution.

The high-frequency resistance of superconducting tin. By H. LONDON. (*Communicated by A. M. Tyndall, F.R.S.—Received* 11 *July* 1940.)

The high-frequency resistance of tin in the superconducting state was measured at a wave-length of 20·5 cm. by a calorimetric method based on the principle of eddy current heating. It was found that the resistance decreases *gradually* when the temperature falls below the transition point in contrast to the *sudden* drop in resistance peculiar to direct currents. An explanation of such a behaviour is given, based on the assumption of the simultaneous presence of normal and superconducting electrons. Good agreement between theory and experiment was found.

Absolute measurements of the conductivity in the normal state at low temperatures with both high and low frequencies were carried out and it was found that at the temperature of liquid helium the conductivity for high frequency is considerably lower than for low frequency. This behaviour is possibly due to the fact that the mean free path of the electrons becomes larger than the penetration depth due to skin effect under the conditions of high conductivity and high frequency.

On the structure of multilayers. I. By G. Knott, J. H. Schulman and A. F. Wells. (*Communicated by E. K. Rideal, F.R.S.—Received* 11 *July* 1940.)

X-ray and microscopic examinations of multilayers of certain long-chain esters deposited on transparent bases have been made. An X-ray photograph of a stationary multilayer of ethyl stearate is apparently identical with that obtained by rotating a single crystal of β-ethyl stearate about the long axis of the cell. The spots previously observed on oscillation photographs of multilayers of ethyl stearate are in reality parts of circles, successive arcs of which appear on increasing the angle of incidence of the X-rays on the multilayer. These facts reveal that the multilayer consists of micro-crystals all oriented with one axis in common, this axis being the normal to the surface of the multilayer. The single crystals appear to have grown without interruption throughout the entire thickness of the multilayer, which in these investigations was about 1000 molecules thick.

On the electromagnetic two-body problem. By J. L. Synge. (*Communicated by E. T. Whittaker, F.R.S.—Received* 11 *July* 1940.)

This paper is classical in the sense that it does not refer to quantum mechanics, but it is relativistic in the sense of the special theory. It contains a direct attack on the problem of the motion of two charged bodies (hydrogenic atom) on the basis of the following hypotheses: (i) the bodies are particles (mathematical points), (ii) the fields are those given by the retarded potential, (iii) the relativistic equations of motion are obeyed, the force being the Lorentz ponderomotive force without a 'radiation term'. These hypotheses appear to be the simplest available and are logically consistent; the dynamical problem based on them seems worthy of investigation, in particular the question as to whether the motion 'degenerates' to an ultimate collision. Previous approximate investigations by Darwin and Sommerfeld did not reveal degeneracy.

A general method of successive approximations leading to an exact solution is outlined. An invariant E and a 4-vector h_r are defined; E reduces to energy and h_r to angular momentum in the Kepler problem, where the mass-ratio is zero. General expressions for the rates of change of E and h_r are obtained, and formulae developed for their principal parts when the mass ratio is small. If the orbit of the light particle is initially approximately circular it remains so, but its radius decreases steadily to an ultimate collision. This agrees with the classical conclusion that an accelerated charge radiates energy, but the rate is much smaller than that given by the Larmor formula. Except in some final calculations, no assumption of small relative velocity is made, the sole basis of approximation being the smallness of the mass ratio.

The kinetics of the thermal decomposition of acetophenone. By C. N. Hinshelwood, F.R.S. and R. E. Smith. (*Received* 15 *July* 1940.)

The mechanisms involved in the thermal decomposition of aldehydes and ketones are varied, and the relations between them somewhat complex. In particular an interesting contrast in behaviour has recently been found between benzaldehyde and acetaldehyde. An investigation of acetophenone has therefore been made for comparison with acetone.

The thermal decomposition of acetophenone takes place predominantly by the step $C_6H_5COCH_3 \rightarrow C_6H_5CH_3 + CO$, the toluene undergoing a subsequent decomposition to give chiefly benzene, methane and carbon. It differs from that of acetone in yielding hardly any ketene. It is homogeneous and nearly of the first order, with no sharp falling off in rate at 20 mm. There is no retardation by nitric oxide or by greatly increased surface, nor can an increased rate of decomposition be induced by the presence of radicals from decomposing diethyl ether. This is taken as evidence for the absence of reaction chains. In this respect the behaviour resembles that of acetone, but other differences in kinetics exist. These are briefly discussed.

The derivation of mechanics from the law of gravitation in relativity theory.

By G. L. CLARK. (*Communicated by Sir Arthur Eddington, F.R.S.—Received* 17 *July* 1940.)

The paper gives a general review of an investigation on some problems on motion in the relativity theory. We begin by discussing the motion of a single particle in a weak gravitational field and obtain both the linear and angular equations of motion; this is followed by brief accounts of the problem of two bodies and that of a rotating rod. In each case considered the equations of motion arise as conditions of integrability of the relativity equations for empty space. Only one of these problems has previously been treated by this method, this being the case of two bodies not connected by a material tension. However, this investigation, which was carried out by Einstein, Infeld and Hoffmann, introduces difficult ideas relating to the use of certain spatial surface integrals; in the present paper we avoid the use of these integrals.

An essential feature of the work is that we take a first order solution for the particular problem considered and substitute this solution in the quadratic terms of $G_{\mu\nu}$, and then build up a second order solution. This method can be employed when we discuss the interior of matter; and, as an example, we conclude the paper by investigating the case of a rotating mass of liquid of constant density.

On the Devonian fish *Palaeospondylus gunni* Traquair.

By J. A. MOY-THOMAS. (*Communicated by E. S. Goodrich, F.R.S.—Received* 29 *July* 1940.)

The anatomy of *Palaeospondylus* has been redescribed and its affinities rediscussed. In the skull the 'hemidomes', 'gammations' and 'Trapezial bars' are shown not to be fused to the neurocranium, but to be visceral articulating elements. The two former are interpreted as parts of the palatoquadrates and the latter as the lower jaws. A hyomandibular and ceratohyal are identified, but the former plays no part in the jaw suspension. The 'rostralia' cannot have formed an anterior ring of cirrhi, because the 'dorsal rostralia' terminate bluntly and may even have formed a continuous plate, and it is concluded that the skull had a prominent rostral prolongation. The presence of paired fins, undoubtedly pelvic and probably also pectoral, is described. The vertebral column and tail previously described upside down are for the first time correctly orientated. The former is shown to have haemal elements throughout its length, and the latter to be heterocercal, the caudal fin being supported by haemal spines and a row of distally bifurcating radials. The view that *Palaeospondylus* has Cyclostome affinities is abandoned on account of the presence of paired fins, centra, a heterocercal tail and jaws. *Palaeospondylus* is believed to be related to the Placoderms (Aphetohyoidea) but to represent a new order the Palaeospondyloidea, which in its lack of dermal skeleton and other features suggests a closer affinity between the Placodermi and Elasmobranchii than is generally held to exist.

The equilibrium-diagram of the system silver-zinc. By K. W. ANDREWS, H. E. DAVIES, W. HUME-ROTHERY, F.R.S. and C. R. OSWIN. (*Received* 3 *August* 1940.)

The equilibrium diagram of the system silver-zinc has been investigated by thermal, microscopic, and X-ray methods. The liquidus curve agrees with that determined by Haycock and Neville (1897) to a very high degree of accuracy. Interesting observations have been made of the decomposition of the β-phase on quenching. Alloys between 45 and 50 atomic % of zinc can be quenched without any visual signs of decomposition, although the work of Owen and Edmunds shows that some of these alloys readily undergo transformation to ordered body-centred cubic structure when quenched. It was found impossible to prevent β-phase alloys containing less than 45 atomic % of zinc from decomposing during quenching, whilst when the zinc content of the β-phase exceeds 50 atomic % of zinc the character of the alloy changes completely, and a number of decomposed or partly decomposed structures are obtained according to the exact conditions of cooling. The $\alpha/\alpha+\beta$-, $\alpha+\beta/\beta$-, and $\beta/\beta+\gamma$-phase boundaries determined in the present work are in general agreement with the results of the earlier workers on this system, and do not confirm the alterations to the diagram recently suggested by Owen and Edmunds who used X-ray methods with quenched filings. The difference is greatest for the $\beta/\beta+\gamma$ boundary and in the present work this has been determined both by microscopic methods and by a new technique involving the use of a high temperature X-ray camera. The remainder of the diagram has been determined in full detail.

The present more accurate determination of the diagram has enabled the following points to be established:

1. The depression of the freezing point of silver by zinc is slightly greater than that produced by equal atomic percentages of indium. This does not confirm the hypothesis of whole number liquidus factors previously suggested by Hume-Rothery, Mabbott and Channel-Evans (1934).
2. The solidus and liquidus curves for the β-phase do not coincide at 50 atomic % of zinc as was proposed by B. G. Petrenko (1929). The freezing range of the equiatomic alloy is approximately $3\frac{1}{2}°$.
3. The determination of phase boundaries by X-ray methods with quenched filings is unreliable for this class of alloy and its limitations are discussed.

The magnetic and other properties of the free electrons in graphite. I.

By K. S. Krishnan, F.R.S. and N. Ganguli. (*Received* 16 *August* 1940.)

The graphite crystal has a large free-electron diamagnetism which is directed almost wholly along its hexagonal axis. Over the whole range of temperature over which measurements have been made, namely from 90 to 1270° K, this free-electron diamagnetism of graphite per carbon atom is found to be equal to the Landau diamagnetism per electron of a free-electron gas obeying Fermi-Dirac statistics and having a degeneracy temperature of 520° K.

From this experimental result it is concluded (*a*) that the number of free or mobile electrons in graphite is just one per carbon atom; (*b*) that the effective mass of these electrons for motion in the basal plane is just their actual mass, showing that the movements in this plane are completely free and uninfluenced by the lattice field; (*c*) that on the other hand their effective mass for motion along the normal to the basal plane is enormous, about 190^3 times the actual mass, which indicates that the mobile electrons belonging to any given basal layer of carbon atoms are tightly bound to the layer, though, according to (*b*), they can migrate quite freely over the whole of the layer; (*d*) that this tight binding accounts for the observed low degeneracy temperature of the electron gas in the crystal.

The electron gas in graphite thus conforms to a simple model which is easily amenable to theoretical treatment, and it has a low degeneracy temperature which is conveniently accessible for experiment. It therefore forms a suitable medium for studying the properties of an electron gas.

The conclusions stated in (2) are in accord with the quantal views of the electronic structure of graphite, and also with its Brillouin zones. There is one zone which can just accommodate three electrons per atom, and the energy discontinuities at all of its boundary surfaces are large. There is a bigger zone which can just accommodate all the four valency electrons, but the energy discontinuities at those of its faces that are perpendicular to the basal plane are very small.

ABSTRACTS

OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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27 November 1940

Ionization, excitation, and chemical reaction in uniform electric fields. IV. A reconsideration of the electron energy balance with special reference to hydrogen.

By R. W. Lunt. (*Communicated by F. G. Donnan, F.R.S.—Received* 26 *June* 1940.)

This paper attempts to formulate in a general way the balance between the gain and loss of energy by electrons for a system in a steady state comprising an electron (and equal positive ion) cloud drifting through a gas at constant pressure in a uniform electric field and confined within a cylindrical insulating boundary co-axial with the field. Such a system serves as a valuable model for the discussion of excitation and chemical reaction in uniform positive column and other discharges. An attempt is thus made to repair the omissions and correct certain errors which are now seen to exist in an earlier treatment of the problem (Part II, Lunt and Meek 1936). In particular, the earlier model is now seen to fail to correspond with a physically realizable steady state, and the conclusions then reached are consequently suspect. These defects are remedied in the new model by the introduction of processes destroying the various species resulting from collisions between electrons and molecules of the parent gas; this involves a detailed consideration of the composition of the gas, especially with regard to the concentration of these secondary species. In illustration, the numerical estimation of the electron energy losses in the principal collision processes in hydrogen is reconsidered; it is found that the earlier conclusions can be supported but in a more precise and extended form.

Ionization, excitation, and chemical reaction in uniform electric fields. V. A reconsideration of Kirkby's investigation of the uniform positive column reaction in electrolytic gas.

By R. W. Lunt, C. A. Meek and G. E. Swindell. (*Communicated by F. G. Donnan, F.R.S.—Received* 26 *June* 1940.)

After reviewing the experimental observations and the theory of Kirkby, an attempt is made to assess the more detailed quantitative theory of excitation as effected by an electron swarm drifting in a uniform electric field (which is developed in earlier papers of this series) by considering how far its application to the particular conditions in Kirkby's experiments provides an explanation for his observations. The present theory of such excitation is a more precise analysis based on Kirkby's ideas, but in its application a number of new considerations are taken into account

It is found that the present theory provides a detailed explanation for the large effect exerted on the rate of reaction by the ratio of the electric field in the positive column to the gas pressure, which effect is Kirkby's main discovery. But it also provides a detailed explanation for certain differences in the rate of reaction between one experiment and another which are revealed by a fresh scrutiny of Kirkby's data, and which he attributed to random experimental errors: the origin of these differences is now found to be ascribable to effects depending on the particular values of the current and the gas pressure selected in the individual experiments.

Furthermore, this detailed explanation of Kirkby's observations is consistent with a number of largely independent experimental data and theoretical considerations, notably: the experimental and theoretical data for single collisions between electrons and hydrogen, oxygen and other molecules, the experimental data for the electron motion in other positive column discharges, the experimental data for the excitation of the continuous spectrum and for the generation of atoms in positive column discharges in hydrogen, the experimental data for the synthesis of ozone in discharges in oxygen, the experimental data and the detailed explanation for the negative glow reaction in electrolytic gas, and the kinetics of the oxidation of hydrogen as based on the experimental data for the reaction in hydrogen-oxygen mixtures as initiated by light absorption.

Wind-tunnel correction for a circular open jet tunnel with a reflexion plate.

By B. Davison and L. Rosenhead, D.Sc. (*Communicated by J. Proudman, F.R.S.—Received* 19 *July* 1940.)

It is advantageous from many points of view to make test-models as large as possible. One method of doing this is to measure the characteristics of half the model in existing wind-tunnels. One half of the aerofoil is mounted horizontally on a vertical reflexion plate and the plate is placed in a suitable position in an open jet which, in the undisturbed state, is of circular section. The contour of the jet is distorted, especially with models of large semi-span, but this distortion is neglected in the analysis. The correcting factor associated with 'uniform' distribution of lift is worked out exactly and that associated with 'elliptic' distribution approximately.

The effect of the induced downwash on the distribution of lift is ignored. The results are given in suitable tables and figures.

Throughout the working range of normal experiments the correcting factor is of the same order of magnitude as that obtaining when a full model is tested in a jet of circular section.

The asymptotic expansion of integral functions. By E. M. Wright. (*Communicated by G. H. Hardy, F.R.S.—Received* 27 *July* 1940.)

1. In a former paper I deduced the asymptotic expansion of the integral function

$$f(x) = \sum_{n=0}^{\infty} \frac{\phi(n)\, x^n}{\Gamma(\kappa n + \beta)} \quad (R(\kappa) > 0)$$

for large x from asymptotic properties of the function $\phi(t)$. In particular, $\phi(t)$ had to be regular and its asymptotic behaviour had to satisfy a certain 'condition A' throughout the half-plane $R(\kappa t) > K$. My results included as special cases most of the known results about the asymptotic expansion of integral functions.

The next step is to widen our class of functions further by relaxing the conditions on $\phi(t)$. I here suppose only that $\phi(t)$ is regular and satisfies condition A in a region of the t-plane including the positive half of the real axis and lying within the half-plane $R(\kappa t) > K$; this region is the 'sector' in which

$$-\mu_1 \leqslant \arg(\kappa t) \leqslant \mu_2, \quad |t| > K, \tag{1}$$

where μ_1, μ_2 are any real numbers satisfying

$$-\tfrac{1}{2}\pi < -\mu_1 \leqslant \gamma \leqslant \mu_2 < \tfrac{1}{2}\pi$$

and $\gamma = \arg \kappa$. From this I deduce the asymptotic expansion of $f(x)$ in that part of the x-plane in which

$$-\min(\mu_1, \omega_2) \leqslant \arg x^{1/\kappa} \leqslant \min(\mu_2, \omega_1), \tag{2}$$

where $\omega_1 = \omega(\mu_1, -\gamma)$, $\omega_2 = \omega(\mu_2, \gamma)$ and $\omega(\mu, \gamma)$ is a real transcendental function of μ, γ and $|\kappa|$. The relevant properties of the function ω are investigated.

The region (2) is bounded by two spirals (degenerating to straight lines if κ is real). If $\mu_2 = \gamma$ or $\mu_1 = \gamma$ the two spirals coincide and the region (2) consists only of the resulting spiral. Hence if $\phi(t)$ is regular and satisfies condition A on the positive half of the real axis, we can find the asymptotic expansion of $f(x)$ on the spiral $\arg x^{1/\kappa} = \gamma$.

If $R(1/\kappa) < \frac{1}{2}$, a number $\mu_0 = \mu_0(\gamma)$ exists such that, if $\mu_2 = \mu_0$ and $\mu_1 = 2\pi R(1/\kappa) - \mu_0$, the region (2) consists of the whole plane and we have the expansion of $f(x)$ for all large x. Since the sector (1) still lies within the half-plane I deduce the results given in a former paper when $R(1/\kappa) < \frac{1}{2}$ from hypotheses substantially less than those of that paper.

I also show that my results are now, in an obvious sense, best possible; that is, if the conditions stated in my theorems are further relaxed, the conclusions are false.

The crystal structure of Rochelle salt (sodium potassium tartrate tetrahydrate). By C. A. Beevers and W. Hughes. (*Communicated by W. L. Bragg, F.R.S.—Received* 1 *August* 1940.)

The complete crystal structure of Rochelle salt (sodium potassium tartrate tetrahydrate) has been determined by Fourier and Patterson methods. Some of the difficulties in the application of these methods are discussed.

The tartrate molecule is found to lie approximately in three planes, the planes of each half of the molecule being inclined at 60° to the plane of the carbon atoms. The tartrate molecules are bonded to sodium and potassium atoms both directly and through the medium of water molecules. If the water molecules are to preserve their customary tetrahedral 'bonding' it is necessary to suppose that one of the carboxyl groups of the molecule is also a dipole. A reversal of the continuous chain of carboxyl-water-water dipoles is a possible explanation of the peculiar dielectric properties of the salt.

The effects of low temperature and acclimatization on the respiratory activity and survival of ram spermatozoa. By Min-Chueh Chang and Arthur Walton. (*Communicated by J. Hammond, F.R.S.—Received* 17 *August* 1940.)

The respiratory activity of ram spermatozoa was measured before and after experimental treatment at low temperature. Sudden cooling has a harmful effect (temperature shock) on the subsequent respiratory activity of the spermatozoa. Temperature shock increases as the temperature is lowered. Temperature shock increases with the time of exposure to the lower temperature. When semen is cooled gradually in stages of 5° C and with an interval between stages, acclimatization of the spermatozoon increases with the length of the interval. If the interval between stages is 2 hr., the semen can be cooled to 1° C and stored at this temperature for 24 hr. without appreciably affecting the subsequent respiratory activity. Rapid warming has no harmful effect upon the subsequent respiratory activity of the spermatozoa. Temperature shock and acclimatization are factors which affect the apparent optimum temperature for the storage of spermatozoa. With rapid cooling the optimum is higher than with slow cooling. Semen cooled gradually to 1° C and stored at this temperature for 6 days retained 74 % of its initial respiratory activity.

The production of cancer by pure hydrocarbons. V. By G. M. Badger, J. W. Cook, F.R.S., C. L. Hewett, E. L. Kennaway, F.R.S., N. M. Kennaway, R. H. Martin and A. M. Robinson. (*Received* 20 *August* 1940.)

Tests for carcinogenic activity have been carried out with some 70 compounds, mostly new substances specially synthesized for the purpose, and related in molecular structure to the known carcinogenic compounds. The results support the view that there is a definite association between carcinogenic activity and molecular structure although the exact limits of structural conditions necessary for carcinogenic activity cannot be precisely defined. Among the compounds related to 1 : 2-benzanthracene and 3 : 4-benzphenanthrene, which comprise the majority of the compounds examined, it is evident that there are certain positions of the molecule in which substitution is especially favourable for carcinogenic properties. In the 3 : 4-benzphenanthrene series, which has not hitherto been extensively investigated, positions 1 and 2 seem to be the favourable positions of substitution.

Comparative tests carried out with stock mice have shown that the potency of a compound in the production of epitheliomas in mice does not always run parallel to its potency in the production of sarcomas by subcutaneous injection. Many compounds have been tested by both modes of administration, and in some cases the power to produce epithelioma was markedly superior to the sarcoma-producing potency, whereas in other cases the reverse was true.

A preliminary study has been made of the incidence of tumours of the lung and of the stomach in mice receiving carcinogenic compounds by the aforementioned two and in some cases a third (oral) method of administration. Very suggestive indications have been found that some of the compounds (the 5-*n*-butyl, 5-*n*-amyl, and 5-*n*-hexyl derivatives in the 1 : 2-benzanthracene series, and the 1- and 2-substituted derivatives as opposed to the 6-, 7- and 8-substituted compounds in the 3 : 4-benzphenanthrene series) are peculiarly effectual in producing tumours of the lung and the stomach. These same groups of compounds are also superior in their capacity to produce epithelial tumours by contrast with their lack of effect on the subcutaneous tissues, and the same is true of the three dibenzfluorenes which have been examined.

On the Bicoecidae: a family of colourless flagellates. By L. E. R. Picken. (*Communicated by J. Gray, F.R.S.—Received* 24 *August* 1940.)

Two distinct species appear to have been confused by James-Clark in his original description of *Bicoeca lacustris*. The two forms have been re-examined and photographed, and it is suggested that the differences between them justify their separation into two species, as suggested by Štolc, the one form to retain the name *B. lacustris*, the other to receive that of *B. vacillans* Štolc.

A new species, *B. maris*, is described, and the affinities of the Bicoecidae, Bodonidae and Craspedomonadidae are discussed.

Molecular structure in relation to oestrogenic activity: derivatives of 4 : 4′-dihydroxydiphenylmethane. By N. R. Campbell. (*Communicated by Sir Robert Robinson, F.R.S.—Received* 26 *August* 1940.)

The oestrogenic activity of four classes of 4 : 4′-dihydroxydiphenylmethane derivatives has been studied. Variation of activity in homologous series has been found, and maximum potencies established. The relationships between structure and maximum potency have been found parallel to those in other series of synthetic oestrogens.

The effect of methyl groups attached to the aromatic rings has been investigated and the results suggest a biological connexion between these groups and groups on the central carbon atom, since the effects vary in marked manner with alteration of the chain length of the central groups.

The apparent sizes of atoms in metallic crystals with special reference to aluminium and indium and the electronic state of magnesium. By W. Hume-Rothery, F.R.S., and G. V. Raynor. (*Received* 4 *September* 1940.)

The interatomic distances in crystals of alloys cannot be accounted for by assigning a fixed atomic radius to each kind of atom, and the causes of this variation are discussed with special references to the Brillouin zone characteristics of different structures. According to the theory of H. Jones, the effect of an overlap across the side of a Brillouin zone is to compress the zone at right angles to the face concerned, and so to expand the crystal lattice in the same direction. This expansion is not a property of an atom which can be transferred to any other structure, but is a characteristic of a structure with sufficient electrons to produce an overlap. The lattice spacings of alloys of aluminium and indium with copper, silver, gold, and magnesium are examined, and the apparent sizes of the aluminium and indium atoms are discussed, and are shown to be in agreement with the theory. The previous suggestion that in metallic aluminium, the atoms exist in an incompletely ionized state is improbable, and is no longer required in order to explain the facts. New experimental data for the lattice spacings of solid solutions of aluminium and indium are presented, and these show that, whilst the curves connecting the a parameter with the composition are smooth and continuous, the corresponding curves for the c parameter show an abrupt change in direction at about 0·75 atomic % of indium or aluminium. This is taken to imply that, although in metallic magnesium with 2 electrons per atom, the overlap of the first Brillouin zone is in the a direction only, the structure is so near to the stage at which the c overlap sets in that the addition of less than 1 electron per 100 atoms causes the c overlap to take place.

The structure of melamine $C_3N_6H_6$. By I. E. Knaggs and K. Lonsdale. With a note on the optical properties of melamine by G. Williams and R. G. Wood. (*Communicated by Sir William Bragg, P.R.S.—Received* 5 *September* 1940.)

Magnetic and X-ray measurements on crystalline melamine, $C_3N_6H_6$, indicate that the molecular formula is of the amide type, based on the cyanuric ring, but that weak hydrogen bridges exist between the NH_2 groups of 1 molecule and the ring nitrogens of others, so that the substance may also tend to behave as an imide. The density is 1·571, the space-group $P\,2_1/a$, and there are 4 molecules in the unit cell. An approximate structure is given. New optical data confirm that this structure is of a layer type, in which respect it is similar to all other cyanuric ring compounds so far examined.

The mobility of positive ions in their own gas. By R. J. Munson and A. M. Tyndall, F.R.S.—(*Received* 9 *September* 1940.)

Values for the mobility of positive ions of neon in neon, argon in argon, krypton in krypton and xenon in xenon have been obtained in pure gas. It is possible to deduce the mobility of an ion of the same mass as the gas atom from the known values for alkali ions in the gas on the assumption that the same mass relationship holds. In each case the observed value is considerably less, the ratio of the two being of the order of 0·60–0·75. This result may be attributed to the phenomenon of electron exchange in the case of ions in their own gas which does not arise with alkali ions.

The variation of mobility with field E and pressure p has been studied. In Kr and Xe there is a marked fall of mobility with increase of E/p, at higher values the velocity of the ion increasing in a linear manner with increase of $(E/p)^{\frac{1}{2}}$.

The mobility of alkali ions in gases. IV. Measurements in gaseous mixtures.

By H. G. DAVID and R. J. MUNSON. (*Communicated by A. M. Tyndall, F.R.S.—Received* 9 *September* 1940.)

The mobilities of some of the alkali ions have been measured in binary mixtures of the inert gases. Blanc's relation that the reciprocal of the mobility is a linear function of the concentration of one of the constituents is accurately obeyed in all cases except for Li^+ in He-Xe mixtures, where the maximum deviation is 4%.

With clustered Li^+ ions in mixtures of water vapour and an inert gas, deviations from this simple relation are observed and increase in magnitude progressively with the density of the gas. These deviations, due to the polar nature of the water molecule, can be explained by assuming a gradual increase in the size of the clustered ion with increase in the percentage of water vapour.

The mobility of alkali ions in gases. V. Temperature measurements in the inert gases.

By K. HOSELITZ. (*Communicated by A. M. Tyndall, F.R.S.—Received* 9 *September* 1940.)

The variation with temperature of the mobility of Li^+ ions in helium, K^+ ions in argon, Rb^+ ions in krypton and Cs^+ ions in xenon has been measured over a considerable range of temperature. Comparison of the results with existing classical theories suggests that the approximate agreement found by Pearce over a similar range of temperature for Cs^+ and Na^+ in He does not hold in general. A quantum mechanical treatment for each individual case seems to be required and for this purpose the experimental data here recorded should be of value.

The distribution of electricity in thunderclouds. II.

By G. D. ROBINSON and Sir GEORGE SIMPSON, F.R.S. (*Received* 10 *September* 1940.)

The investigation of the distribution of electricity in thunderclouds described by Simpson and Scrase in 1937 has been continued at Kew Observatory. Alti-electrographs, which record the sign of the potential gradient, are sent up on small free balloons. Additional observations made during eight thunderstorms in the years 1937, 1938 and 1939 are discussed and the conclusions reached by Simpson and Scrase are confirmed. Each thundercloud has positive electricity in the upper half of the cloud, negative electricity in the lower half and in most storms if not in all there is a concentrated positive charge below the main negative charge. The generation of the positive and negative charges in the main body of the cloud is ascribed to the impact of ice crystals and that of the positive electricity in the base of the cloud to the breaking of rain drops in an ascending current of air.

Exchange effects in the theory of the continuous absorption of light. I. By D. R. Bates and H. S. W. Massey, F.R.S. (*Received* 10 *September* 1940.)

The continuous absorption coefficients of normal Ca and Ca^+ are calculated. Both the discrete and continuous wave functions used in the calculation include the effect of electron exchange. Comparison is affected with results obtained neglecting exchange and it is found that appreciable modifications are introduced by its inclusion. This is particularly true for Ca^+ which is a sensitive case owing to very strong interference in the integrand of the transition matrix element. The bearing of the results on the calculation of absorption coefficients in general is discussed and it is pointed out that the discrepancy between theory and experiment for potassium arises because this also is a very sensitive case.

An application of the results for Ca is made to resolve a discrepancy between determinations of interstellar electron densities carried out using different methods by Strömgren and Struve.

The polarization of electrons by double scattering. By H. S. W. Massey, F.R.S., and C. B. O. Mohr. (*Received* 10 *September* 1910.)

The scattering of electrons by gold, xenon and krypton atoms has been investigated, using Dirac's equations. The polarization to be expected by double scattering at 90° has been studied for an energy range from 100 to 150,000 eV. The results agree substantially in the energy range 10,000–150,000 eV with those obtained by Mott for an unscreened gold nucleus. It is found, however, that for lower energies the effect of screening is more important. In particular at energies for which the intensity of single scattering at 90° is near a minimum, a large polarization is to be expected in the case of gold. For xenon the polarization never exceeds 4 % and for krypton 2 %.

Modifications of the interaction between a nucleus and electron which would reduce the polarization are considered.

As an example of a field as different as possible from the Coulomb type, the polarization produced in double scattering by a potential well is investigated in detail.

Application of the coincidence method for measurements of short life periods. By Józef Rotblat. (*Communicated by J. Chadwick, F.R.S.—Received* 16 *September* 1940.)

A method is described for the determination of short radioactive and nuclear life periods. It consists in varying the length of the electrical impulses produced in a coincidence arrangement of two Geiger-Müller counters. The method can be used for the measurement of life periods in the range 10^{-7} to 10^{-1} sec. The half-life period of radium C′ has been measured in this way and found to be $1{\cdot}45(\pm 0{\cdot}05) \times 10^{-4}$ sec. Experimental evidence has been obtained for the absence of γ-rays in the transition radium C′ → radium D, and some conclusions regarding the lifetimes of excitation states of radium C′ have been drawn. The determination of the intensity of weak sources and of the efficiency of Geiger counters for various types of radiation is described. The effect of a time lag in Geiger counters on the measurement of coincidences is discussed.

The respiratory function of the haemoglobin of the earthworm. By M. L. Johnson. (*Communicated by H. Munro Fox, F.R.S.—Received* 17 *September* 1940.)

The oxygen consumption of earthworms (*Lumbricus herculeus* Savigny) has been measured at 10° C, in the dark, in atmospheres containing 20, 10, 5, 2·5 and 1 % of oxygen (i.e. at partial pressures of oxygen of about 152, 76, 38, 19 and 8 mm. mercury) with and without the addition of enough carbon monoxide to saturate the haemoglobin of the blood.

In the absence of carbon monoxide the rate of oxygen consumption was the same at 152 and 76 mm.; below 76 mm. it fell sharply.

The rate of oxygen consumption of carbon monoxide-treated worms was lower than that of normal worms at oxygen pressures of 152, 76, 38 and 19 mm. This lowering of rate was not due to the effect of carbon monoxide on respiratory enzymes, for the oxygen consumption of sliced earthworm tissue was not lowered by the amounts of carbon monoxide used in the experiments on whole worms. It is concluded therefore that haemoglobin acts as an oxygen carrier at atmospheric partial pressure of oxygen and at lower oxygen pressures down to 19 mm.

The amount of oxygen carried by the haemoglobin was probably greatest when the pressure of oxygen was about 76 mm. mercury. It is deduced that the loading pressure of earthworm haemoglobin may be as high as 76 mm., and is certainly higher than 19 mm.

The haemoglobin of the blood was responsible for supplying about 20 % of the respired oxygen when the oxygen pressure was at 152 mm., 35 % at 76 mm., 40 % at 38 mm., and 20 % at 19 mm. of oxygen.

The characteristics of thermal diffusion. By S. Chapman, F.R.S. (*Received* 26 *September* 1940.)

When a gas-mixture is contained in a vessel in which a steady temperature gradient is maintained, a concentration gradient is in general set up, whose amount is determined by the logarithm of the temperature ratio, and by k_T, the thermal diffusion ratio; the general theory of non-uniform gases gives successive approximations to k_T, and the first of these, $[k_T]_1$, is accurate within a few per cent. The paper discusses the dependence of $[k_T]_1$ on (*a*) the ratio of the molecular masses; (*b*) their concentration ratio (c_1 or c_2); (*c*) the two ratios of the molecular diameters, inferred from the coefficient of viscosity, to their joint diameter, inferred from the coefficient of diffusion; and (*d*) three parameters depending on the mode of interaction between the unlike molecules. When this interaction is according to the inverse-power law, the three parameters (*d*) are all expressible in terms of the mutual force-index, and $[k_T]_1$ is a function of five independent variables. The general nature of its dependence on these variables is discussed, with particular reference to the end values (for c_1

or c_2 zero) of the thermal diffusion factor α, given by k_T/c_1c_2; these end values involve fewer variables (less by two) than the general values, and their functional character can be represented graphically. It is shown that k_T may be zero not only when c_1 or c_2 is zero, but also for at most one intermediate mixture-ratio. Formulae for $[k_T]_1$ appropriate to various special cases are also given.

An investigation of the disintegration of boron by slow neutrons.

By R. S. WILSON. (*Communicated by J. Chadwick, F.R.S.—Received 27 September* 1940.)

The disintegration of boron by slow neutrons has been investigated using an ionization chamber filled with boron trichloride in conjunction with a linear amplifier.

The magnitudes of the ionization impulses, measured by the oscillograph deflexions, should give under appropriate conditions a measure of the energy release in the disintegration process. The various factors which can affect the size of the oscillograph deflexion are discussed so as to define the appropriate conditions of experiment.

Evidence is found for two disintegration energies. Assuming that the greater energy release corresponds to the formation of the Li^7 nucleus in the ground state and is therefore 2·99 MeV, then the smaller energy, which is released in about 93–94 % of the disintegrations, is 2·57 ± 0·05 MeV. An explanation is offered of the contradiction with the results of Maurer and Fisk. The γ-radiations associated with the reaction have been detected and a rough measurement of the quantum energy has been made.

Molecular anisotropy of urea and of related compounds.

By K. LONSDALE. (*Communicated by Sir William Bragg, P.R.S.—Received* 4 *October* 1940.)

The magnetic anisotropy of urea has been measured and compared with that of the carbonate and nitrate groups. It does not appear that the hydrogen bonds contribute appreciably to the anisotropy; the latter indicates about the same degree of resonance in all three groups. The anisotropy of the refractivities is greater in urea than in the nitrate group, and considerably greater than in the carbonate group, showing that the $(NH_2)^-$ radical must be much more polarizable than the O^{--} ion, allowance being made for the interionic distances. Urea nitrate, urea oxalate, cyanuric acid dihydrate and anhydrous cyanuric acid all crystallize in layer structures, as shown by cleavage, X-ray data, magnetic and optical observations. The magnetic anisotropy of urea nitrate and urea oxalate is approximately additive, but that of cyanuric acid dihydrate is considerably larger than the additive value for three urea molecules, being comparable with that of other cyanuric ring compounds. Although the molecules in each layer must be joined together by hydrogen bonds, it does not follow that hydrogen bonds form the links between successive layers, since the layer to layer distance of 3·05 ± 0·10 A is characteristic even of cyanuric triazide, which contains no hydrogen bonds.

Submarine seismic investigations. By E. C. BULLARD and T. F. GASKELL. (*Communicated by Sir Gerald Lenox-Conyngham, F.R.S.—Received 9 October* 1940.)

The refraction seismic method has been used to investigate the form of the surface of the hard rocks underlying the sediments on the continental shelf to the west of the English Channel. This surface is found to slope steadily downwards on receding from the land, and to reach a depth of over 8000 ft. at the 100 fm. line. The velocity of elastic waves in the sediments is about 6000 ft./sec. near the surface and up to 9700 ft./sec. lower down; compared to 16,000–22,000 ft./sec. in the basement. The bearing of these results on the structure and history of the shelf is discussed.

The theory of the divisions in Saturn's rings. By G. R. GOLDSBROUGH, F.R.S. (*Received 9 October* 1940.)

In an earlier paper on this subject the author (1921) proposed a theory of the rings which showed satisfactory agreement with the observed measurements of the rings. The mathematical method was, however, subjected to criticism. In the present paper the subject is again attacked by an entirely different method which is free from the objections raised against the first method.

A family of periodic orbits of the particles forming the ring, when perturbed by a satellite, is constructed, and the stability of these orbits is examined by the method of small displacements. Stability determined in this way is shown to have a real meaning when applied to the problem in hand.

The positions of instability of the particles lead to the divisions in the ring and the inner and outer boundaries, close agreement with observation being obtained. The analysis, though quite different from that of the earlier paper, reproduces its main features, and introduces further points of interest.

Observations on the chemical constitution of galactogen from *Helix pomatia*: *l*-galactose as a component of a polysaccharide of animal origin. By E. BALDWIN and D. J. BELL. (*Communicated by Sir F. Gowland Hopkins, O.M., F.R.S.—Received* 15 *October* 1940.)

The polysaccharide galactogen, isolated from the albumin gland of *Helix pomatia*, has been shown to be built up from both *d* and *l* isomers of galactose in the probable proportions of 6:1. Crystalline derivatives of *dl* galactose have been obtained from the polysaccharide, thus affording what is believed to be the first proof of the presence of *l*-galactose components in a substance of animal origin. The products of methanolysis of methylated galactogen have been analysed; they are composed of tetramethyl methylgalactosides and dimethyl methylgalactosides in molecular proportions closely agreeing with the simple ratio of 4:3. The former material consists of a mixture of one molecule of 2:3:4:6-tetramethyl methyl-*l*-galactosides with three molecules of 2:3:4:6-tetramethyl methyl-*d*-galactosides, while the dimethyl methylgalactosides consist entirely of the 2:4-dimethyl-*d*-derivatives. Possible structures for the chemical molecule of galactogen are discussed.

The fine structures of phloem fibres. II. Untreated and swollen jute.

By R. D. Preston. (*Communicated by W. T. Astbury, F.R.S.—Received 16 October* 1940.)

Studies of the jute fibre by X-ray methods, under the polarizing microscope, and after swelling with various reagents, lead to the conclusion that the optical heterogeneity of the wall is not to be explained in terms of changes in cellulose chain direction. The evidence available shows that the wall is composed of chains forming a single, steep spiral. The wall spiral becomes steeper as the cell elongates and this continues until the onset of wall thickening.

The fibre in many ways provides a study complementary to that of hemp. The outer layers of the wall are the least lignified and have the least resistance to swelling. The phenomenon of 'ballooning' is therefore never observed, though structures resembling balloons may be produced by a special technique. For similar reasons transverse cracks are never observed during the swelling process.

Impregnation with lignin seems to cause an increase in thickness of the wall even in the outer layers which are far removed from the cytoplasm-wall interface.

The results fully confirm the conclusions already reached that observations of swollen material are usually entirely misleading.

Radio echoes and cosmic ray showers.

By P. M. S. Blackett, F.R.S., and A. C. B. Lovell. (*Received* 22 *October* 1940.)

It is suggested that the origin of some of the transient ionic clouds, generally assumed to be responsible for the low level sporadic radio reflexions, may be due to large cosmic ray showers.

It is shown that cascade cosmic ray showers of sufficient energy to produce some of these radio reflexions certainly exist, but there is insufficient published evidence to decide whether any of the echoes already observed are actually due to such showers. More conclusive evidence could be obtained from the frequency-size distribution of the radio echoes observed from a horizontal or vertically directed beam.

ABSTRACTS

OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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31 December 1940

The seasonal variations of cosmic-ray intensity and temperature of the atmosphere.

By A. Duperier. (*Communicated by P. M. S. Blackett, F.R.S.—Received* 4 *March* 1940.)

A careful examination of the upper atmospheric data for Europe and the United States indicates that the mean temperature of the upper atmosphere in spring differs from that of summer more than from that of winter. The magnitude of the second difference, as defined, depends on the height of the atmosphere which is considered and is a maximum at a height of about 6 km. and changes sign at heights above 12 km. (figure). This lag in the warming of the atmosphere in spring is found to be paralleled by a lag in the diminution of intensity of the cosmic rays. A similar phenomenon is found in autumn. The cooling of the atmosphere as a whole is found to be less between summer and autumn than between autumn and winter, though the effect is markedly less definite than in spring.

The cosmic ray variation is found to be correlated more closely with the mean temperature of the atmosphere up to 16 km. than with the temperature near the ground. This provided additional support for the theory of Blackett that the temperature variation of penetrating cosmic rays is related to the instability of the mesotron. The temperature coefficient of the cosmic rays as deduced from the seasonal data is found to be 0·18 % per ° C, and this is in rough agreement with the prediction of the theory.

The production of waves by the sudden release of a spherical distribution of compressed air in the atmosphere. By J. J. Unwin, M.Sc. (*Communicated by G. I. Taylor, F.R.S.—Received* 27 *June* 1940.)

An attempt has been made to develop a method for dealing with solutions of problems connected with the production of waves by spherical concentrations of compressed air. Starting from the general equations for three-dimensional spherically symmetrical flow in a homogeneous compressible medium having constant entropy everywhere, a process has been devised to apply step-by-step calculations over small intervals of time to investigate the general features of such a motion. A complete solution has been worked out in one particular case for a not very intense initial distribution of pressure, and various indirect checks have indicated that the results are reasonably accurate. These results show many features of distinct interest. As distinct from plane or spherical sound wave theory, it is found that a train of waves passes away from the centre of disturbance, the amplitudes and wave lengths falling off from wave to wave. Furthermore, as distinct from finite amplitude plane wave theory which shows that any wave must eventually become a shock wave, the waves obtained in the finite amplitude spherical wave case show no indication of becoming shock waves, and indeed show towards the closing stages of the calculation a similarity to sound wave propagation. The method is applicable to any spherically symmetrical motion up to such a time as the formation of a shock wave takes place and then fails owing to the assumption of constant entropy.

Short period fluctuations in the characteristics of wireless echoes from the ionosphere. By T. L. Eckersley, F.R.S. and F. T. Farmer. (*Received* 6 *August* 1940.)

This paper deals with the effect of irregularities in the ionosphere on reflected radio waves. Such irregularities occur mainly in the E layer. These irregularities vary rapidly and cause e.m.f.s on two spaced aerials to be incorrelated with a consequent apparent spread in direction. Manual methods are not sufficiently rapid to resolve these variations. These rapid fluctuations are studied by recording the ellipses on a cathode-ray tube produced by the e.m.f.s on two spaced frames. This is done by taking photographs at intervals of $\frac{1}{25}$ sec., and the technique is made applicable to impulses by a device which desensitizes the apparatus for all but a small fraction of the time base period, so that observations can be confined to a single pulse of the echo pattern. The same apparatus can be used both for directional and polarization measurements by appropriate connexion of the aerials.

The results show that for normal E and F reflexions the relative e.m.f. in the two aerials, and in consequence the directions and polarizations, remain constant over many seconds.

Reflexions from scattering clouds are however entirely different. The apparent ray directions and the polarizations only remain constant over a fraction of a second (between $\frac{1}{25}$ and $\frac{1}{2}$ sec.) and the difficulty of balancing by manual methods the electric force in one frame against that in another is entirely explained. The results show that the abnormal E region is also irregular in time and space.

The dissociation constants of the methylammonium ions and the basic strengths of the methylamines in water. By D. H. Everett and W. F. K. Wynne-Jones. (*Communicated by Sir Harold Hartley, F.R.S.—Received* 4 *November* 1940.)

The dissociation constants of the ions $CH_3NH_3^+$, $(CH_3)_2NH_2^+$ and $(CH_3)_3NH^+$ have been determined at intervals of 10° from 0 to 50° by a method involving the measurement of the potential of a hydrogen electrode placed in buffer solutions of each amine and its hydrochloride.

By combining these results with the known values for the ionic product of water, values have been obtained for the ionic equilibria of the type

$$CH_3NH_2 + H_2O = CH_3NH_3^+ + OH^-$$

conventionally referred to as the ionization constants of the amines.

The heats, entropies and heat capacities of these reactions have been calculated and the results compared with the simple electrostatic theory of the ionization process: it is found that the heat capacity changes are too large to be explained on this basis.

The kinetics of the polymerization of isoprene on sodium surfaces. By J. L. Bolland. (*Communicated by E. K. Rideal, F.R.S.—Received* 4 *November* 1940.)

The kinetics of the polymerization of isoprene liquid and vapour occurring on sodium surfaces have been investigated at 60 and 25° C. In the case of the liquid phase, polymerization diffusion of monomer to the catalytic surface is shown to be the rate controlling factor, except when the sodium surface is in the form of a sphere of sufficiently small dimensions.

The kinetics of the polymerization in presence of toluene are consistent with the view that polymeric chains are initiated by formation of free radicles on the sodium surface, the subsequent propagation occurring while the polymer is still attached to the sodium: termination occurs exclusively by interaction with toluene. In absence of toluene an alternative termination reaction, leading to cross linking and requiring 4·6 kcal. more activation energy than the process involving toluene, comes into play.

On the salinity of the surface waters of the Irish Sea. By J. Proudman, F.R.S. (*Received* 4 *November* 1940.)

This paper gives a statistical treatment of most of the observations of the salinity of the surface waters of the Irish Sea which were taken up to the end of the year 1939. Its objects are: (1) To put on record the chief variations of the salinity in the central part of the sea during the whole period of observation. (2) To calculate grand mean values of the characteristics of the salinity and of its seasonal variation for stations distributed over the whole area of the sea. (3) To investigate the degree of correlation between the salinities at pairs of stations, and to find for what time differences the coefficients of correlation attain maximum values. (4) To investigate the degrees of correlation between the salinities at different stations and the rainfall and barometric gradients; and to find the time lags which correspond to maximum correlation coefficients. (5) To obtain from the correlation coefficients such indications as they may afford of the mean currents of the sea.

The heart of the salamander (*Salamandra salamandra*), with special reference to the conducting (connecting) system and its bearing on the phylogeny of the conducting systems of mammalian and avian hearts.

By F. Davies and E. T. B. Francis. (*Communicated by W. E. Le Gros Clark, F.R.S.—Received* 8 *November* 1940.)

The study of nine salamander hearts by means of serial sections cut in three planes has demonstrated muscular continuity between the several cardiac chambers, and the entire absence of any specialized muscle or 'nodal tissue' at the junctional sites, or in any other part of the heart. The heart muscle forms a continuum. The cardiac muscle fibres are characterized by their large size (i.e. breadth) and they have the same general histological characters in all parts of the heart.

In each of the chambers the musculature is arranged in a basket-work fashion, but at each of the junctional sites the muscle suddenly changes to a regular circular arrangement. The sinus, at its junction with the right atrium, contains striated muscle only in its ventral wall, and it is this wall only of the sinus which thus establishes muscular continuity with the ring of muscle (S.-A. ring) around the sinu-atrial opening. The musculature of both atria is continuous with that of the ventricle in two ways. From the ring of muscle (A.-V. ring) surrounding the common opening of the atria into the ventricle, the atrio-ventricular funnel dips down into the ventricle, and the caudal border of this funnel is continuous with an invaginated part of the base of the ventricle, and more extensively with ventricular papillary muscles, which are continued into the inner ventricular trabeculae about the middle level of the ventricle. The A.-V. funnel is homogeneous in structure. The ventricular muscle is directly continued into that of the bulbus cordis.

The course which the wave of contraction takes during its transmission from the sinus throughout the heart and many of the details of the phases of the cardiac cycle are described.

The delay in the transmission of the wave of contraction from one cardiac chamber to the next is accounted for by the relatively long path which the impulse has to traverse at the junctional sites, without postulating the existence of specialized 'block fibres' at these sites. The branching of the muscle fibres has the effect of converting the morphological circular arrangement of the fibres at these junctions into a physiological spiral.

The glycogen content of the various parts of the frog's heart is found to increase in the order sinus, atria, ventricle and bulbus cordis. This is correlated with a similar increasing order of density of musculature and work done, the glycogen being a reserve potency for the energy of muscular contraction. The fact that the intrinsic rhythmic rates of the several chambers decrease in the same order as the glycogen content increases may or may not be coincidental.

Cutting and ligature experiments, with cinephotographic and kymographic records, reveal the intrinsic rhythmic rates of the various cardiac chambers of the salamander heart.

No satisfactory reason has yet been adduced to account for the different intrinsic rhythmic rates of the several parts of the heart when these are isolated from each other.

The dorsal mesocardium has been traced in its entirety. The sinu-ventricular fold is a part of the continuous dorsal mesocardium and does not constitute a direct muscular sinu-ventricular connexion.

The distribution of the intracardiac nerve cells has been noted and the probable pathway of migration of these nerve cells in the embryo has been suggested.

The significance of the results of this investigation in relation to the phylogeny of the specialized conducting system of the hearts of homoiothermal vertebrates (mammals and birds) is discussed. The view is expressed that the cardiac conducting systems of homoiothermal vertebrates constitute a neomorphic development, correlated with functional requirements, and are not remnants of more extensive tissues of similar structure in the lower vertebrate heart. Variations in this newly evolved formation probably account for the different descriptions of such elements in various mammalian and avian hearts.

Anatomical and histological changes during the oestrous cycle in the mare.

By J. Hammond, F.R.S. and K. Wodzicki. (*Received* 18 *November* 1940.)

The mares chosen for the investigation of the changes in the reproductive organs during the oestrous cycle were kept under observation for some time before they were killed. The duration of the heat period in these animals was 7 days and the length of the dioestrus was 16 days. Ovulation takes place at about a day before the end of oestrus. The growth of the follicle is very rapid during the period of oestrus. The size of the ovary during the oestrous cycle is chiefly influenced by the growing Graafian follicle. The number of follicles present at different stages varies greatly. The numerous small follicles present at the beginning of oestrus disappear later in the cycle; it is suggested that this may be due to the lack of follicle-stimulating hormone.

The colour of the corpus luteum varies greatly at different stages of the cycle. The rupture of the follicle is associated with some bleeding. The active stage of the corpus luteum is very short and the maximum diameter of the corpus luteum seems to be always below that of the Graafian follicle. The greater development of the Graafian follicle, with its secretion of oestrin, in the mare leads to its playing a more important role than in the cow and the sow, in which species the corpus luteum takes a more dominant part in the cycle. It appears that the much longer oestrus in the mare than in the cow is due to the longer time required by the follicle to come to the surface and to break through. This is probably due to the peculiar structure of the ovary in the mare, since the ovulation, which is spontaneous, can only occur in the small ovulation fossa. No pronounced secretion stage occurs during oestrus in the Fallopian tubes.

The anatomical and histological changes in the uterus of the mare during the cycle are slight as compared with those in species which have a specialized placentation, such as the rabbit, ferret and cat; they are similar to those changes which occur in a species with a simple placentation such as the pig.

Cyclic changes are described in the epithelial cells of the cervix, which acts as a large mucous gland. It is probable that ejaculation takes place directly through the cervix into the uterus.

The changes in the vagina proper and the vestibulum vaginae are described; they are less pronounced in the mare than in the cow, but some cornification and leucocytosis, similar to that found in some rodents, occurs.

Structure and molecular anisotropy of sorbic acid,

$CH_3.CH:CH.CH:CH.COOH$.

By K. LONSDALE, J. M. ROBERTSON and I. WOODWARD. (*Communicated by Sir William Bragg, P.R.S.—Received* 18 *November* 1940.)

A preliminary study of sorbic acid has shown that the monoclinic unit cell contains eight molecules, these being linked in pairs by hydrogen bonds about the symmetry centres of the crystal, with the long chain axis of the molecule in or near to the (010) plane and inclined at between 10 and 15° to the *a* axis in the obtuse angle *β*. The molecular chains must be rotated so as to bring some of the atoms out of the (010) plane, and an approximate measure of this rotation is provided by measurements of optical and magnetic anisotropy, which give ±35° as the angle between the *b* axis and the normal to the molecular plane. The magnetic anisotropy due to resonance in the conjugated chain is about half as large as that in the benzene ring and falls into line with previous observations on non-cyclic conjugated compounds. The orientation of the molecules has been confirmed by observation of the size and shape of the diffuse spots occurring on well-exposed Laue photographs taken with radiation from a copper target, and the usefulness of these photographs as a secondary method of structure determination has been emphasized.

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